TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1027

MEDICA - ODONTOLOGICA

GENERATION AND CHARACTERIZATION OF KNOCKOUT MICE AND ANALYSIS OF PATIENT MATERIAL DEMONSTRATES A ROLE FOR TISSUE INHIBITOR OF METALLOPROTEINASES 4 (*Timp4*) IN THE CARDIOVASCULAR SYSTEM AND TUMOR METASTASIS

by

Ilpo Koskivirta

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

From the Department of Medical Biochemistry and Genetics and
Turku Doctoral Programme of Biomedical Sciences,
University of Turku,
Turku, Finland

Supervised by

Professor Eero Vuorio
Department of Medical Biochemistry and Genetics
University of Turku

and

Docent Hannu Järveläinen
Department of Medicine
Turku University Hospital

Reviewed by

Professor Yrjö T. Konttinen
Department of Medicine
University of Helsinki

and

Professor Seppo Nikkari
Department of Medical Biochemistry
University of Tampere

Dissertation opponent

Professor Andrew H. Baker
Institute of Cardiovascular and Medical Sciences
University of Glasgow

ISBN 978-951-29-5097-3 (PRINT)
ISBN 978-951-29-5098-0 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2012

To my family

ABSTRACT

Ilpo Koskivirta. Generation and Characterization of Knockout Mice and Analysis of Patient Material Demonstrates a Role for Tissue Inhibitor of Metalloproteinases 4 (*Timp4*) in the Cardiovascular System and Tumor Metastasis (2012).
Department of Medical Biochemistry and Genetics, University of Turku, 20520 Turku, Finland.

This thesis focuses on tissue inhibitor of metalloproteinases 4 (TIMP4) which is the newest member of a small gene and protein family of four closely related endogenous inhibitors of extracellular matrix (ECM) degrading enzymes. Existing data on TIMP4 suggested that it exhibits a more restricted expression pattern than the other TIMPs with high expression levels in heart, brain, ovary and skeletal muscle. These observations and the fact that the ECM is of special importance to provide the cardiovascular system with structural strength combined with elasticity and distensibility, prompted the present molecular biologic investigation on TIMP4.

In the first part of the study the murine *Timp4* gene was cloned and characterized in detail. The structure of murine *Timp4* genomic locus resembles that in other species and of the other *Timp*s. The highest *Timp4* expression was detected in heart, ovary and brain. As the expression pattern of *Timp4* gives only limited information about its role in physiology and pathology, *Timp4* knockout mice were generated next. The analysis of *Timp4* knockout mice revealed that *Timp4* deficiency has no obvious effect on the development, growth or fertility of mice. Therefore, *Timp4* deficient mice were challenged using available cardiovascular models, i.e. experimental cardiac pressure overload and myocardial infarction. In the former model, *Timp4* deficiency was found to be compensated by *Timp2* overexpression, whereas in the myocardial infarct model, *Timp4* deficiency resulted in increased mortality due to increased susceptibility for cardiac rupture. In the wound healing model, *Timp4* deficiency was shown to result in transient retardation of re-epithelialization of cutaneous wounds. Melanoma tumor growth was similar in *Timp4* deficient and control mice. Despite of this, lung metastasis of melanoma cells was significantly increased in *Timp4* null mice. In an attempt to translate the current findings to patient material, TIMP4 expression was studied in human specimens representing different inflammatory cardiovascular pathologies, i.e. giant cell arteritis, atherosclerotic coronary arteries and heart allografts exhibiting signs of chronic rejection. The results showed that cardiovascular expression of TIMP4 is elevated particularly in areas exhibiting inflammation.

The results of the present studies suggest that TIMP4 has a special role in the regulation of tissue repair processes in the heart, and also in healing wounds and metastases. Furthermore, evidence is provided suggesting the usefulness of TIMP4 as a novel systemic marker for vascular inflammation.

Key words: TIMP, MMP, knockout mice, pressure overload, myocardial infarction, cardiac rupture, wound healing, metastasis, atherosclerosis, giant cell arteritis

LYHENNELMÄ

Ilpo Koskivirta. Poistogeenisen *Timp4*-hiirimallin tuottaminen ja kuvaaminen sekä potilaiden kudosnäytteiden tutkiminen osoittaa *Timp4*:llä olevan merkittävä asema sydän- ja verisuonisairauksissa ja melanooman etäpesäkemuodostuksessa (2012).
Lääketieteellinen biokemia ja genetiikka, Biolääketieteen laitos, Turun yliopisto, 20520 Turku.

Tämän tutkimuksen kohteena on TIMP4 (väliaineen metalloproteinaasien kudosinhibiittori 4), joka on sidekudoksen väliainetta pilkkovien entsyymien luonnollisten inhibittoreiden geeni- ja proteiiniperheen tuorein jäsen. Tutkimuksen alussa käytettävissä oleva tietous TIMP4:stä viittasi sen ilmentymisen rajautuvan lähinnä sydämeen, aivoihin, munasarjoihin ja luustolihakseen. Nämä havainnot ja sidekudoksen väliaineen erityinen tärkeys sydän- ja verisuonijärjestelmän rakenteen elastisuuden ja venytyskestävyyden ylläpitäjänä olivat perusteena tälle molekyylibiologiselle TIMP4-tutkimukselle. Työn ensimmäisessä osassa kloonattiin hiiren *Timp4*-geeni ja kuvattiin sen yksityskohtainen rakenne. Hiiren *Timp4*-geenilokus on samankaltainen muiden tunnettujen lajien ja muiden *Timp*-geenilokusten kanssa. *Timp4*-geenin ilmentyminen oli määrällisesti merkittävintä sydämessä, munasarjoissa ja aivoissa. Koska geenin ja proteiinin esiintyminen kudoksessa antaa vain rajallista informaatiota sen fysiologisesta ja patologisesta merkityksestä, työn toisessa vaiheessa tuotettiin *Timp4*-poistogeenisiä hiiriä. Näiden hiirten tutkiminen osoitti, ettei *Timp4*:n puutoksella ole havaittavaa vaikutusta hiiren kehitykseen, kasvuun tai lisääntymiskykyyn. Sen vuoksi *Timp4*-poistogeenisiä hiiriä altistetiin kokeellisille sydän- ja verisuonisairausmalleille. Sydämen painekuormitusmallissa *Timp4*:n puutos kompensoitui *Timp2*:n ilmentymisen lisääntymisellä. Sydäninfarktimallissa *Timp4*:n puutos altisti sydämen kammioseinämän repeämiselle ja lisäsi täten kuolleisuutta. Työn kolmannessa osassa *Timp4*-puutteen osoitettiin hidastavan ihohaavan paranemista epitelisaatiovaiheessa, vaikkei haavan lopullinen paraneminen häiriintynytkään. Melanoomasolujen kasvu *Timp4*-poistogeenisillä ja kontrollihiirillä ei eronnut toisistaan. Tästä huolimatta melanooman etäpesäkkeet keuhkoissa lisääntyivät merkittävästi *Timp4*-poistogeenisillä hiirillä. Työn viimeisessä osassa selvitettiin tulosten lääketieteellistä merkittävyyttä tutkimalla TIMP4:n ilmentymistä tulehduksellisissa sydän- ja verisuonisairauksissa: jättisoluarteriitissa, ateroskleroottisissa sepelvaltimoissa ja sydänsiirrännäisten hylkimisreaktioiden yhteydessä. Tulokset osoittivat TIMP4:n osallistuvan keskeisesti sydän- ja verisuonisairauksien tulehdusreaktioon.

Tutkimuksen tulokset antavat viitteitä siitä, että TIMP4:llä on tärkeä asema kudoksen paranemistapahtuman säätelyssä sydämessä ja ihohaavoissa ja että sillä on osuutensa myös metastaasien muodostumisessa. Jatkotutkimukset ovat kuitenkin aiheellisia ennen kuin TIMP4:ä voidaan mahdollisesti hyödyntää ihmissairauksien diagnostiikassa ja hoidossa.

Avainsanat: TIMP, MMP, poistogeeninen hiirimalli, sydäninfarkti, haavan paraneminen, melanooma, etäpesäkkeet, jättisoluarteriitti, ateroskleroosi

TABLE OF CONTENTS

ABBREVIATIONS

aa	amino acid
AB	aortic banding
ADAM	a disintegrin and metalloproteinase
ADAM-TS	a disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	one-way analysis of variance
bp	base pair(s)
(c)DNA	(complementary) deoxyribonucleic acid
C-	carboxy
Col	collagen
COMP	cartilage oligomeric matrix protein
COPD	chronic obstructive pulmonary disease
CSF1	colony stimulating factor 1, macrophage colony-stimulating factor
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EMB	endomyocardial biopsy
EMT	epithelial to mesenchymal transition
EPA	erythroid-potentiating activity
ES cell	embryonic stem cell
EST	expressed sequence tag
FGF2	fibroblast growth factor 2
GCA	giant cell arteritis
GPI	glycosylphosphatidylinositol
HGF	hepatocyte growth factor
kb	kilobase(s)
kDa	kilodalton
LDL	low-density lipoprotein
LPS	lipopolysaccaride
LRP	low-density lipoprotein related protein
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblast
MI	myocardial infarction
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
N-	amino-
NCBI	National Center for Biotechnology Information
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RECK	reversion-inducing cysteine-rich protein with Kazal motifs
RT-PCR	reverse transcriptase polymerase chain reaction

SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SYN	synapsin
TACE	tumor necrosis factor converting enzyme
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyltransferase-mediated nick end labeling
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their respective Roman numerals:

I Otto Rahkonen, Ilpo Koskivirta, Sanna Oksjoki, Eero Jokinen and Eero Vuorio (2002). Characterization of the murine *Timp4* gene, localization within intron 5 of the synapsin 2 gene and tissue distribution of the mRNA. **Biochim Biophys Acta** 1577(1):45–52.

II Ilpo Koskivirta*, Zamaneh Kassiri*, Otto Rahkonen, Riku Kiviranta, Gavin Y. Oudit, Trevor D. McKee, Ville Kytö, Antti Saraste, Eero Jokinen, Peter P. Liu, Eero Vuorio, and Rama Khokha (2010). Mice with tissue inhibitor of metalloproteinases 4 (*Timp4*) deletion succumb to induced myocardial infarction but not to cardiac pressure overload. **J Biol Chem** 285(32):24487-93.
*The first two authors contributed equally

III Ilpo Koskivirta, William Cruz-Muñoz, David Smookler, Mervi Toriseva, Sanna Oksjoki, Veli-Matti Kähäri, Eero Vuorio and Rama Khokha (2012). *Timp4* deficiency does not affect melanoma cell growth *in vivo* but increases propensity for melanoma metastasis. *Manuscript.*

IV Ilpo Koskivirta, Otto Rahkonen, Mikko Mäyränpää, Sari Pakkanen, Michael Husheem, Annele Sainio, Harri Hakovirta, Jukka Laine, Eero Jokinen, Eero Vuorio, Petri Kovanen and Hannu Järveläinen (2006). Tissue inhibitor of metalloproteinases 4 (TIMP4) is involved in inflammatory processes of human cardiovascular pathology. **Histochem Cell Biol** 126(3):335-42.

In addition, some unpublished data are included in this thesis. The original publications have been reproduced with the permission of the copyright holders.

1. INTRODUCTION

This thesis focuses on tissue inhibitors of metalloproteinases (TIMPs) which in mammals form a small gene and protein family of four closely related members. All TIMPs exhibit a wide tissue expression pattern with TIMP4 being the most restricted with a high expression in tissues such as heart, brain, ovary and skeletal muscle. TIMPs 1, 2 and 4 are soluble proteins while TIMP3 is bound to extracellular matrix (ECM). The most important biological function of TIMPs is to serve as endogenous inhibitors of matrix metalloproteinases (MMPs) and related enzymes.

Regarding MMPs, they form a large family of 25 zinc dependent proteases that can degrade all ECM components. The ECM which is produced by different types of connective tissue cells provides the mammalian body with structural strength combined with elasticity, distensibility (e.g. in vascular wall), resistance to compression and resilience (e.g. in cartilage), supportive and filtration functions in basement membrane etc. Collagens are the best known components of the different types of the ECM although a number of proteoglycans and glycoproteins characteristic to these tissues are also known. The role of MMPs becomes critically important in their capability to degrade native collagen molecules which are resistant to most other proteolytic enzymes.

However, the ECM is also an important regulator of cell behaviour by different ways. It functions as storage for growth factors and other bioactive molecules. Furthermore, ECM molecules as such and their degradation products interact with cell surface receptors regulating cellular functions through various signalling cascades. Thus, in addition to its structural role the ECM also plays essential roles in the determination, differentiation, proliferation, survival, polarity and migration of cells.

Physiologically ECM turnover is an integral part of development and growth in every organ. In some tissues, such as bone, continuous ECM turnover takes place to renew the tissue structure, whereas in other adult tissues (e.g., cartilage) the turnover of collagenous ECM is very slow. In pathology, ECM turnover is involved for instance in inflammation, malignancies, atherosclerosis and in heart failure. ECM turnover is controlled by a tightly controlled balance of production of ECM components and their degradation which is initiated by MMPs. Another important control point in matrix turnover occurs at the level of the regulation of MMP activity. This is where TIMPs come into play.

In vitro, all TIMPs can inhibit most MMPs except TIMP1 that cannot inhibit membrane-type MMPs (MT-MMPs). At any specific time, each tissue possesses a unique composition of ECM components, specific MMPs (some of which are active, others inactive) and TIMPs. Thus, biochemical characterization of tissues gives only limited information about the roles of individual MMPs or TIMPs in physiology or pathology. *In vivo* models are clearly needed to characterize the biological roles of individual components of the MMP-TIMP system in physiological and pathological degradation of the ECM.

The technology to produce knockout mouse models provides a unique tool for studying the role of specific gene-protein systems *in vivo*. Generation and characterization of *Timp1*, *Timp2* and *Timp3* null mice has greatly expanded the knowledge of the roles of TIMPs in development and in pathologies. All *Timp* knockout mice are born healthy and develop normally. *Timp1* deficient mice exhibit reduced fertility and resistance to bacterial infections. *Timp2* null mice show reduced activation of proMMP2, but they exhibit behaviour and motor phenotypes. *Timp3* knockout mice develop emphysematic lung disease and heart failure by ageing.

When this study was started TIMP4 was the least characterized of the TIMPs. Earlier work had suggested TIMP4 to be the most characteristic for the cardiovascular system. Downregulation of cardiac *Timp4* had been observed in heart failure and after myocardial infarction, while *Timp4* overexpression had been reported after experimental vascular injury. As functions of TIMP4 still remained poorly known, the aim of this study was to generate and characterize *Timp4* knockout mice. As the first step towards these experiments, murine *Timp4* gene was cloned and characterized in detail. After generation and initial characterization of *Timp4* deficient mice, these mice were subjected to experimental cardiac pressure overload and myocardial infarction. In addition, wound healing, malignant growth and tumor metastasis, processes where the roles of MMPs and TIMPs are better understood, were studied in *Timp4* deficient mice. To expand our knowledge about TIMP4 in the vasculature and human pathology, TIMP4 expression was studied in coronary atherosclerosis and other inflammatory disorders of human cardiovascular system.

2. REVIEW OF THE LITERATURE

2.1. Extracellular matrix

The extracellular matrix (ECM) provides tissues with a three-dimensional structural scaffold but it is also essential for regulating different cellular processes. It is not only present in connective tissue and basement membranes but forms an integral part of almost all tissue types. The best known function of the ECM is provision of structural support, not only in skeletal structures (e.g. bone, cartilage, ligaments, tendons), but also in blood vessels, skin and essentially all parenchymal tissues. In addition to structural support, the ECM also contributes to other physicochemical properties of tissues, such as elasticity, distensibility and resilience.

The ECM contains classes of macromolecules such as collagens, elastin, and microfibrillar proteins, proteoglycans including hyaluronan, and noncollagenous glycoproteins (Järveläinen et al, 2009). The structural properties of tissues differ widely because they are composed of different combinations of specific ECM components. Functionally, the components of the ECM can also be classified into structural proteins (certain collagens and elastin), adhesive proteins (laminin, fibronectin, type IV and VI collagens), anti-adhesive proteins or matricellular proteins (tenascin, thrombospondin and osteopontin) and proteoglycans (Jane-Lise et al, 2000). Each group contains multiple members expressed in a tissue-specific manner; thus considerable diversity is seen in the structure of the ECM between different tissues.

Collagens are the most important structural components of the ECM and also the most abundant protein family in the human body (Aumailley & Gayraud, 1998; Myllyharju & Kivirikko, 2004). The main function of collagens in any mammalian body is the provision of structural support. To perform this function, collagens are notoriously resistant to proteolysis by common proteolytic enzymes, but can be degraded by specific proteinases as will be described below. Collagens are homo- or heterotrimeric molecules of constituent α chains. Three α chains are folded together into a triple helix to form a basic structure of the collagens. Today, 28 different types of collagens are known in vertebrates (Bateman et al, 2009). Collagens are subdivided into fibril-forming collagens (types I, II, III, V, XI, XXIV and XXVII) and a heterogenous group of nonfibrillar collagens (Table 2.1). The latter can be further separated into several subgroups: collagens associated with banded fibrils including FACITs, (fibril-associated collagens with interrupted triple helix, collagen types IX, XII, XIV, XX), FACIT-like collagens (types XVI, XIX, XXI and XXII) and type VII collagen, which forms anchoring fibrils for basement membranes; network-forming collagens (types IV, VI, VIII and X) including type IV collagen, known as a basement membrane collagen; collagens with transmembrane domains (collagen types XIII, XVII, XXIII and XXV); the family of type XV and XVIII collagens, which can be cleaved to generate antiangiogenic

peptides (restin, also known as endostatin-XV; and endostatin(-XVIII), respectively); and collagen types XXVI and XXVIII, which do not fit to the previous groups (Gordon & Hahn, 2010). In addition, a large number of other proteins (e.g. a subcomponent C1q of complement, adiponectin, collectins, ficolins, three macrophage receptors) containing triple-helical collagenous domains have been characterized (Myllyharju & Kivirikko, 2004). However, these molecules have not been defined as collagens, as their function does not appear to be linked to connective tissues. However, criteria for distinguishing between a collagen and a protein containing a collagenous domain(s) are not clear.

Table 2.1. Collagen types, their tissue distribution and functions

	Tissue distribution	**Functions**
Fibrillar collagens		
I	Most connective tissues	Structural component of all tissues except cartilage
II	Cartilage, vitreous humor, cornea	Predominant component of cartilage, mediates interactions with proteoglycans
III	Extensible connective tissues, e.g. skin, lung, muscle, vascular system; dominant collagen type in granulation tissue	Structural component
V	Tissues containing collagen I	Structural component of basement membrane, interacts with type I collagen, inhibits endothelial cell adhesion and proliferation
XI	Cartilage, tissues containing collagen II	Structural component of articular cartilage, modulates cartilage matrix homeostasis
XXIV	Developing bone and cornea	
XXVII	Cartilage, eye, ear and lung	Suggested to play a role during the transition of cartilage to bone[1]
Collagens associated with banded fibrils		
IX	Cartilage, tissues containing collagen II	Structural component; interacts with collagen type II
XII	Tissues containing collagen I	Structural component; interacts with other matrix components
XIV	Tissues containing collagen I	Structural component; interacts with other matrix components
XX	Corneal epithelium, skin, cartilage and tendon	Col 1 -domain disrupted in human gene, thus unlikely functions as collagen in humans
XVI	Many tissues	Component of microfibrillar apparatus and of heterotypic cartilage fibrils[2]
XIX	Hippocampal neurons, rhabdomyosarcoma cells	Required for the formation of hippocampal synapses[3]
XXI	Many tissues	
XXII	Tissue junctions	

	Tissue distribution	Functions
VII	Basement membranes	Anchoring fibrils for basement membranes; interacts with other ECM components
Network-forming collagens		
IV	Basement membranes	Structural component of basement membrane
VI	Most connective tissues	Structural component; mutations cause muscle diseases
VIII	Many tissues, especially endothelium	Structural component of ECM, involved in stabilization of membranes and angiogenesis
X	Hypertrophic chondrocytes	Structural component; regulates matrix mineralization and compartmentalization of ECM components
Transmembranous collagens		
XIII	Many tissues	Structural component of connective tissue, interacts with other matrix components
XVII	Skin hemidesmosomes	Structural component of cutaneous basement membrane; involved in cell-matrix adhesion
XXIII	Metastatic tumor cells, lung, cornea, brain, skin, tendon, kidney[4]	
XXV	Neurons	Implicated in pathogenesis of Alzheimer's disease[5]
Endostatin precursor collagens		
XV	Many tissues	Structural component of basement membrane; acts as structural organizer of ECM, inhibition of angiogenesis and tumor growth
XVIII	Many tissues (especially liver and kidney)	Structural component of basement membrane; inhibition of angiogenesis and tumor growth
Other collagens		
XXVI	Testis, ovary	
XXVIII	Peripheral nerves, skin, calvaria[6]	

Modified from Prockop and Kivirikko (1995); Myllyharju and Kivirikko (2004); Järveläinen et al. (2009); Gordon and Hahn (2010).

[1] Hjorten et al, 2007
[2] Kassner et al, 2004
[3] Su et al, 2010
[4] Koch et al, 2006
[5] Tong et al, 2010
[6] Veit et al, 2006

As discussed above, the ECM also contains a variety of *noncollagenous proteins*, which have variable structural functions. Many of them are critical for the assembly and stability of collagen fibers, and in addition are able to participate in binding of growth factors and thereby in the regulation of cellular functions.

Elastin is the major ECM component responsible for flexibility. It is secreted as a monomer, tropoelastin, which is organized into elastin polymers. These are further organized into either elastic fibers or as in vascular wall into elastic lamellae (Wagenseil & Mecham, 2009). Elastin is abundant in tissues such as lung, skin and blood vessels (especially large arteries), where elasticity is a fundamental feature of tissue. Other *microfibrillar proteins* include *fibrillin*, *fibulin* and *emilin*. They function as structural and regulating components in microfibrils and basement membranes. Fibrillin 1 is the major structural component of extracellular microfibrils and its mutations cause Marfan syndrome, a heritable disorder characterized by disorganization of elastic fibers and dysregulation of transforming growth factor-β (TGF-β) (Judge & Dietz, 2005). Typical manifestations of Marfan syndrome include skeletal anomalies, such as elongated limbs and joint laxity, various eye pathologies, valvular heart diseases and dilatation of aortic root, which is associated with increased risk of aortic dissection (Gray & Davies, 1996).

Proteoglycans are macromolecules constituted of a core protein and attached glycosaminoglycan (GAG) side chains. Carbohydrate units of the latter are sulphated and include chondroitin, dermatan, keratan and heparan sulphates. However, *hyaluronan*, the major carbohydrate component of the ECM, is a large unsulfated GAG without a core protein (Chen & Abatangelo, 1999). Proteoglycans can be classified based on the size of their core protein or types of GAG chains. Proteoglycans with large core proteins are important structural components of the ECM and they include *aggrecan*, *versican*, *brevican* and *neurocan* (Aumailley & Gayraud, 1998; Järveläinen et al, 2009). *Decorin*, *biglycan*, *fibromodulin* and several other members comprise a large family of *small leucine-rich proteoglycans* (SLRPs), which function as structural proteins, bind and modulate growth factors such as TGF-β and also regulate intracellular phosphorylation (Schaefer & Iozzo, 2008). Furthermore the proteoglycan family contains members (agrin, bamacan, perlecan) that are found in basement membranes (Järveläinen et al, 2009).

Noncollagenous ECM glycoproteins include *fibronectins*, *laminins*, *SPARC* (secreted protein, acidic and rich in cysteine), *tenascins*, *thrombospondins* and *nidogen*/*entactin* (Järveläinen et al, 2009).

2.1.1 Functions of the extracellular matrix

The extracellular matrix forms an organized network of the macromolecules providing structural support for organs and tissues, for cell layers in the form of basement membranes and for some single cells as substrates for migration (Hynes, 2009). Different properties of connective tissues can be explained by their different composition of ECM components which provides a huge number of combinations with subsequent variability

in function. For practical purposes extracellular matrices can be viewed as supramolecular structures of different ECM components. ECM molecules interact with cells through adhesion receptors, mainly integrins, contributing to cellular processes such as adhesion, migration, polarity, differentiation, proliferation and survival (Hynes & Naba, 2012). Also physical forces can be transmitted into cytoskeleton through integrin connections of the ECM components. Moreover, the ECM regulates cellular processes by its ability to bind and release growth factors. Furthermore, ECM proteins as such or their degradation products are also capable of binding cell surface receptors other than integrins.

Functions of the ECM are largely dependent on the supramolecular complexes. In different tissues, unique constitution of the ECM contributes to the functions of the specific organ. Cardiac myocytes surrounded by a dense extracellular network fibrillar collagens (mainly types I and III) form the myocardium. The cardiac ECM supports the three-dimensional organ structure and is essential for transforming mechanical contractions of single myocytes to the pump function serving the whole circulatory system. The arterial wall is composed of three layers: the innermost layer called the intima, the media and the adventitia which is the outermost layer (Wagenseil & Mecham, 2009). The luminal surface of the intima is lined with endothelial cells. The media is comprised of elastic lamellae, other ECM components and smooth muscle cells that are mainly responsible for the production of the ECM of the large and medium-sized muscular arteries. The main ECM components, i.e. elastin, type I and III collagens, of the medial layer provide arterial wall with its unique mechanical properties storing and releasing energy during cardiac cycle. The outermost layer, the adventitia, contains a collagen-rich ECM, which in large arteries is for the most part responsible for maintaining the integrity under high pressure conditions (Wagenseil & Mecham, 2009).

The importance of the ECM proteins is evident by the number of severe inherited connective tissue disorders that are caused by mutations in ECM genes. Three different mechanisms have been characterized contributing to pathologies: 1) secreted mutant protein can disrupt ECM interactions and compromise ECM structure and stability; 2) secretion of the ECM components can be reduced by mutations affecting synthesis or causing cellular retention or degradation; 3) mutant misfolded ECM proteins can cause endoplasmic reticulum stress leading to altered gene expression and even to apoptosis (Bateman et al, 2009).

Human diseases caused by ECM gene mutations can affect a large range of organs and the severity of the disease varies according to the specific mutation of the patient. Mutations of *COL1A1* and *COL1A2* genes (which encode the two different polypeptide chains of the type I procollagen, a proα1(I)$_2$proα2(I) heterotrimer) can cause osteogenesis imperfecta, a genetic disorder characterized by fragile bones, abnormal teeth and weak tendons (Byers et al, 2006). In Alport syndrome, a disease leading to progressive renal failure, several mutations have been characterized in different genes encoding polypeptides forming type IV collagen (Kashtan, 1999; Myllyharju & Kivirikko, 2001). Ehlers-Danlos syndrome is a heterogenous group of disorders manifested as joint laxity, skin hyperextensibility or fragility of the large arteries (Beighton et al, 1998). Ehlers-

Danlos syndrome is caused by genetic mutations leading to abnormal production or processing of fibrillar collagens. Mutations of the microfibrillar protein fibrillin 1 are linked to Marfan syndrome, a disorder leading to overgrowth of long bones and to defects of the heart valves and aorta (Gray & Davies, 1996; Judge & Dietz, 2005).

2.2 Extracellular matrix turnover

Earlier, the ECM was regarded as a relatively stable fibrous network. Today, the ECM is known to be under constant remodeling including production of new ECM molecules, assembly of fibrous structural proteins and modification and degradation of ECM components by different proteases (Daley et al, 2008). The rate of ECM remodeling varies in different tissues and under different physiologic and pathologic situations. As already mentioned, degradation of native collagen fibrils presents a specific challenge in ECM turnover, as these structures are remarkably resistant to most proteolytic enzymes. Therefore, enzymes belonging to two protease families of matrix metalloproteinases (MMPs) and adamalysins (ADAMs and ADAMTS) play a central initiating role in ECM degradation. The action of these two enzyme families is supported by serine proteases and cysteine proteases. Under physiological conditions, during embryogenesis, growth and tissue repair, ECM degradation is tightly regulated while in tumor growth and metastasis and many other pathologic states dysregulated ECM degradation is a common finding (Hutchinson et al, 2011; Lu et al, 2012).

2.3 Matrix metalloproteinases (MMPs)

A collagenolytic activity was characterized for the first time in tadpole tissues undergoing metamorphosis by Gross and Lapiere in 1962. The first human collagenase was purified from rheumatoid synovium (Woolley et al, 1975). In 1986, a cDNA for human collagenase (MMP1) was cloned from fibroblasts (Goldberg et al, 1986). The transcript resembled a cDNA expressed in rat fibroblasts published a year earlier and was later named as rat MMP3 (Matrisian et al, 1985).

Today, matrix metalloproteinases (also called matrixins) comprise a gene family of 25 related members in vertebrates. 24 of them are found in humans including the duplicated *MMP23* gene (Nagase et al, 2006). Thus, 23 different MMPs exist in humans. The murine MMP family resembles that of humans except MMP1, which has two putative murine homologues, Mcol-A (Murine collagenase-like A) and Mcol-B (Balbín et al, 2001). However, the expression pattern or substrate specificity of neither of them is identical with human MMP1. Vertebrate MMPs are numbered from 1 to 28. MMPs 4, 5, 6 and 22 are absent because they were later found to be identical to previous members; and MMP18 (Collagenase4 in *Xenopus laevis*) is not found in mammals.

MMPs are either secreted or, in case of membrane-type MMPs (MT-MMPs), bound to the cell surface. In addition, intracellular forms of MMP1, MMP2 and MMP11, have been reported and they may cleave intracellular substrates (Nagase et al, 2006). Except

for the intracellular splice variants, all MMPs are synthesized as pre-proenzymes and the signal peptide is removed during translation. MMPs consist of at least a pro-domain (~80 aa) and a catalytic domain (~170 aa). All but MMP7, MMP23 and MMP26 have also a proline-rich linker peptide called a hinge region and a hemopexin-like domain in the C-terminal end. Length of the hinge region varies between MMPs possibly contributing to substrate specificity (Murphy & Knäuper, 1997; Nagase et al, 2006). The hemopexin domain can mediate binding to substrates thus regulating substrate specificity, but in specific circumstances it also mediates binding to the non-inhibiting, i.e. C-terminal domain of TIMPs as described later for proMMP2 and TIMP2 (Itoh & Seiki, 2006).

The pro-domain of MMPs contains a conserved PRCGXPD sequence called a cysteine switch and the catalytic domain contains a zinc binding motif HEXXHXXGXXH (Nagase et al, 2006). Three histidines in the zinc binding motif are responsible for the binding of a catalytic zinc ion (Zn^{2+}) to the active site. Altogether, the catalytic domain contains two zinc ions (one catalytic and one structural), and up to three calcium ions stabilizing the structure. In proMMPs, binding of a cysteine of the propeptide prevents a water molecule needed for catalysis from binding to the catalytic zinc. The catalytic domain also contains a characteristic Met-turn, a methionine residue downstream of the zinc binding site, which supports the structure around the catalytic zinc. The zinc binding site and the Met-turn are conserved in the metzincin group of metallopeptidases containing also subfamilies of adamalysins (ADAM, ADAMTS and class III snake venom proteins), astacins (BMP1/TLL proteins and meprins) and bacterial serralysins (Bode et al, 1993). Later, a MEROPS peptidase database has classified MMPs into the M10A subfamily and adamalysins into the M12B subfamily of MA metallopeptidases (http://merops.sanger.ac.uk (Rawlings et al, 2012)).

2.3.1 Regulation of MMP activity

The activity of MMPs is regulated at several levels; gene expression, compartmentalization, pro-enzyme activation and enzyme inactivation (for reviews see Hadler-Olsen et al, 2011; Page-McCaw et al, 2007; Ra & Parks, 2007). In normal adult tissues, MMPs are usually expressed at negligible or low levels, while in physiologic or pathologic situations involving processes such as tissue remodeling and inflammation, significant MMP expression is typically present. All MMPs contain a pro-domain, which keeps the proMMP inactive. Activation of the proMMP is achieved by proteolysis of the pro-domain or by modification of the thiol group of a cysteine residue in the pro-domain. For example oxidants produced by leucocytes can activate MMPs through oxidation of the thiol group. Proteolytic activation of proMMPs can be achieved through other MMPs; for example proMMP2 is activated on the cell surface by active MT1-MMP in a process involving also another MT1-MMP, which is inhibited through TIMP2 bound to its active site. Also other proteases, like serine protease plasmin, possibly activate MMPs through a proteolytic cleavage of the pro-domain. Several MMPs including all MT-MMPs contain a furin cleavage site between the pro-domain and the catalytic domain. These MMPs can be activated intracellularly

by pro-protein convertases or furins. MT-MMPs are not the only MMPs localized on the cell surface, and a regulated pericellular localization (i.e. compartmentalization) is likely to be important for many MMPs. MMP2 can be bound to the αvβ3-integrin, MMP1 to the α2β1-integrin, MMP9 to CD44 and MMP7 to cell surface proteoglycans (Parks et al, 2004). These interactions might be involved in localized activation of MMPs or co-localization of the enzyme and its substrate. As each MMP has somewhat unique affinity for different substrates, the protease activity present in a tissue is partly determined by the concentrations of available MMPs as well as potential substrates.

A significant level of MMP regulation takes place at the level of enzyme inactivation. Active MMPs can be inactivated by natural or synthetic inhibitors or through proteolytic processing (Tallant et al, 2010). The most important natural inhibitors are tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site of MMP forming a 1:1 stochiometric inactive complex. α2-macroglobulin is present in the circulation and can trap many different types of enzymes including MMPs and mediate their clearance by scavenger receptors (Nagase et al, 2006). RECK (reversion including cysteine-rich protein with kazal motifs) is a membrane-bound glycoprotein, which inhibits MMP2, 9 and 14 (Muraguchi et al, 2007; Oh et al, 2004a; Oh et al, 2001). Typical synthetic MMP inhibitors, such as batimastat and marimastat, chelate zinc ions effectively and thereby inactivate MMPs (Hu et al, 2007). They are not especially specific, but act on most metalloenzymes (Newby, 2012; Peterson, 2006). As mentioned earlier, oxidants produced by inflammatory cells can activate proMMPs. However, oxidation of amino acids needed for catalytic activity can also result in inactivation of an active MMP.

2.3.2 Classification of MMPs

MMPs are frequently classified into six groups based on their substrate specificities and domain composition: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others.

The *Collagenase* group comprises MMP1, MMP8 and MMP13, which all are capable of initiating degradation of native interstitial collagens (types I, II and III) by degrading them into typical 1/4 and 3/4 fragments. They are also able to cleave some other types of collagens and certain components of the ECM such as aggrecan, gelatin and fibronectin (Visse & Nagase, 2003). Moreover, collagenases are capable of degrading non-matrix proteins such as interleukin-8, pro-tumor necrosis factor-α, protease-activated receptor-1 and several insulin-like growth factor binding proteins (Fanjul-Fernández et al, 2010). MMP8 is mainly expressed in neutrophils. It has been shown to modulate tumor cell adhesion and invasion suppressing metastasis (Gutiérrez-Fernández et al, 2008), and its deficiency increases susceptibility to chemical induced skin tumors (Table 2.2) (Balbín et al, 2003). Two major cartilage ECM components, collagen type II and aggrecan, are substrates for MMP13 *in vivo*; and it is needed for a normal endochondral ossification in mice (Inada et al, 2004; Stickens et al, 2004). MMP2 and MT1-MMP can also cleave interstitial collagens, but they are classified into other groups based on their domain organization.

Gelatinases A (MMP2) and B (MMP9) contain an insertion of three fibronectin type II repeats in their catalytic domain, which mediates their binding to gelatin (Murphy et al, 1994). In addition to gelatin, gelatinases can cleave other ECM components such as type IV, V and XI collagens, laminin and aggrecan. Similarly to collagenases, MMP2 digests fibrillar collagens (Aimes & Quigley, 1995). Although the collagenolytic activity of MMP2 is weaker than that of MMP1 in solution, it might be physiologically important near the cell surface, because the activation of proMMP2 is mediated by a complex of proMMP2, MT1-MMP and TIMP2 on the cell surface as will be discussed below. In addition to extracellular space, MMP2 has been localized in cardiac myocytes along myofibrils, in mitochondria and in nuclei (Kwan et al, 2004). In mice, lone MMP2 deficiency results in slower gain of body weight, abnormal long bone and craniofacial development and articular cartilage destruction (Itoh et al, 1997; Mosig et al, 2007). However, mice with combined deficiency of MMP2 and MT1-MMP are lethal at birth with respiratory failure, abnormal blood vessels and immature muscle fibers (Oh et al, 2004b). MMP9 null mice exhibit abnormality in growth plate vascularization and ossification (Vu et al, 1998). Later, gelatinase knockout mice have been subjected to numerous experimental challenges (Table 2.2). These studies suggest important and many times different roles for MMP2 and MMP9 in such processes as tumor progression, metastasis, inflammation and cardiovascular diseases (Fanjul-Fernández et al, 2010; Hu et al, 2007).

Stromelysins (MMP3, MMP10 and MMP11) possess a similar domain composition as collagenases, but they are not capable of cleaving fibrillar collagens. Stromelysins can cleave other ECM components such as laminin and type IV collagen as well as other molecules such as E-cadherin and latent TGF-β1 (Parks et al, 2004). They are able to cleave proMMPs thus activating latent enzymes (Fanjul-Fernández et al, 2010). MMP3 deficiency results in impaired mammary gland branching morphogenesis (Table 2.2) (Wiseman et al, 2003). MMP10 null mice have not an obvious phenotype, but they develop a more severe pulmonary infection when infected with *Pseudomonas aeruginosa* (Kassim et al, 2007). MMP11 is structurally different from other stromelysins and it is also a weaker protease of ECM molecules (Murphy et al, 1993). Like all MT-MMPs and MMP28 (see below), MMP11 contains a furin cleavage site between the pro- and catalytic domains, and is activated intracellularly and secreted as an active enzyme (Pei & Weiss, 1995). In MMP11 deficient mice, neointima formation is accelerated after vascular injury (Lijnen et al, 1999).

Matrilysins (MMP7 and MMP26) are the smallest MMPs as they lack a hemopexin domain in contrast to all other MMPs except MMP23 (Nagase et al, 2006). MMP7 is constitutively expressed in adult epithelial cells in several tissues. Along with ECM components, it can cleave cell surface molecules, for instance pro-α-defensins, Fas-ligand, latent TNF, syndecan-1 and E-cadherin (Parks et al, 2004). In tracheal wounds, MMP7 is required for a normal re-epithelialization (Table 2.2) (Dunsmore et al, 1998). MMP7 participates also in mucosal defense against bacteria as well as in regulation of different chemokines and cytokines contributing to inflammatory cell influx and activation (Parks et al, 2004). MMP26 is expressed in a few normal tissues such as uterus, placenta and kidney, and in several epithelial carcinomas.

Membrane-type MMPs (MT-MMPs) comprise six MMPs, which are anchored to cell surface through a transmembrane domain (MMP14, MMP15, MMP16 and MMP24), or through a glycosylphosphatidylinositol anchor (GPI; MMP17 and MMP25) attached to the hemopexin domain by a short linker (Nagase et al, 2006). All MT-MMPs contain a furin cleavage site and can thus be activated by proprotein convertases like the Golgi-associated furin and expressed as active enzymes on cell surface. Among several cell surface and extracellular substrates, MT1-MMP cleaves native interstitial collagens similarly to collagenases (Holmbeck et al, 1999; Seiki, 2003). In contrast to other MMP knockout mice, MT1-MMP deficiency leads to severe phenotype characterized by impaired collagenolytic activity, bone deformation and premature death (Table 2.2) (Holmbeck et al, 1999). All MMPs with a transmembrain domain have been suggested to activate proMMP2 (Yana & Seiki, 2002). At least, MT1-MMP (MMP14) can also activate proMMP13 (Knäuper et al, 1996). GPI-anchored MT-MMPs (MMP17 and MMP25) have unique features that separate them from MT-MMPs with a transmembrain domain: they possess a more limited ECM substrate profile, they cannot promote proMMP2 activation, they are inhibited by all the TIMPs and also by clusterin (also known as apolipoprotein J), a multifunctional and ubiquitously expressed protein (Sohail et al, 2008). Among the MMPs, MT-MMPs possess some unique regulatory functions on the cell surface (Itoh & Seiki, 2006). First, MT-MMPs can be autocatalytically processed into inactive variants. Secondly, MT-MMPs can be endocytosed from the cell surface and also recycled back to the surface. Thirdly, MT-MMPs can be shed form the cell surface into the extracellular space.

MMP12, MMP19, MMP20, MMP21, MMP23, MMP27 and MMP28 are not usually categorized into any of the previous groups. Macrophage metalloelastase (MMP12) is an essential elastin degrading protease and it is essential for macrophage migration (Shipley et al, 1996). In mice, MMP12 deficiency protects from cigarette smoke-induced emphysema (Hautamaki et al, 1997). Furthermore analyses of single nucleotide polymorphisms in the *MMP12* gene support the importance of MMP12 in chronic obstructive pulmonary disease (COPD) in humans (Hunninghake et al, 2009). MMP19 can degrade many components of the ECM including aggrecan and cartilage oligomeric matrix protein (COMP) (Stracke et al, 2000a; Stracke et al, 2000b). It is widely expressed in various tissues including vascular wall. MMP19 is capable of digesting plasminogen and thus generating angiostatin-like fragments (Brauer et al, 2011). MMP20 (enamelysin) digests amelogenin. It is expressed during tooth development and is required for a normal tooth enamel development (Caterina et al, 2002). MMP21 was first found in Xenopus and later also in mice and humans (Ahokas et al, 2002; Marchenko et al, 2003). MMP23 has unique structural features including a cysteine-rich and an immuglobulin-like domain, lacking a hemopexin domain and lacking the cysteine switch of the pro-domain. As MMP23 has a transmembrane domain at the N-terminal end of its propeptide it is considered to be a type II membrane protein. However, when the propeptide is cleaved, also the anchor to the cell surface is lost and thus MMP23 is not usually grouped with the MT-MMPs (Pei et al, 2000). MMP27 was originally found in chicken embryo fibroblasts and shown to digest gelatin and casein (Yang & Kurkinen, 1998), but its possible *in vivo* functions are poorly

known. Epilysin (MMP28) is highly expressed in the human testis and at lower levels in several tissues such as epidermis, lung, heart, colon, intestine and brain (Lohi et al, 2001). Increased MMP28 expression has been observed in cartilage of patiens with osteoarthritis or rheumatoid arthritis (Illman et al, 2008). Recently generated MMP28 null mice exhibit no obvious phenotype, but in a murine model of *Pseudomonas aeruginosa* pneumonia, MMP28 deficient mice exhibited accelerated macrophage recruitment, enchanced bacteria clearance and reduced neutrophilia in the lungs (Manicone et al, 2009).

Box 2.1. Human hereditary diseases linked to specific MMPs

Characterization of MMPs *in vitro* and in animal models has revealed important roles for some MMPs. The developmental and tissue specific roles of MMPs have been revealed at least partially using gene-targeted animal models. Another approach towards this purpose has been to identify and characterize MMP-related diseases. So far, four different hereditary diseases have been linked to specific mutations in MMP genes (according to the Mammalian Degradome Database, http://degradome.uniovi.es/)

Torg-Winchester syndrome is an autosomal recessive syndrome characterized by a common feature of osteoporosis or osteolysis in hands and feet and variable features of generalized osteoporosis and multiple subcutaneous nodules. In genetic studies, different mutations of MMP2 gene have been characterized in families carrying the disease (Martignetti et al, 2001; Rouzier et al, 2006; Zankl et al, 2005; Zankl et al, 2007). When serum samples of the affected patients have been analyzed by zymography, a complete loss of MMP2 activity has been demonstrated (Martignetti et al, 2001; Zankl et al, 2007).

Metaphyseal anadysplasia is a group of disorders characterized by an early-onset regressive dysplasia of distal metaphyses of long bones and femoral necks (Maroteaux et al, 1991). The anomalies disappear or their severity decreases after the age of 2 years. A milder recessive form of the syndrome is caused by homozygous mutation causing loss of function of either MMP9 or MMP13, while a more severe dominantly-inherited form is caused by missense mutations in the prodomain of MMP13 causing autoactivation of MMP13 and intracellular degradation of both MMP13 and MMP9 (Lausch et al, 2009).

The spondyloepimetaphyseal dysplasias (SEMD) comprise a heterogenous group of skeletal disorders featuring defective growth and modelling of vertebrae and of epiphyses and metaphyses of long bones. The Missouri type of SEMD, inherited as an autosomal dominant form and featuring moderate to severe metaphyseal changes, mild epiphyseal involvement and pear-shaped vertebrae in childhood, has been linked to a mutation of MMP13 gene (Kennedy et al, 2005). The mutation leads to intracellular autoactivation and degradation of the mutant proenzyme and secretion of proteolytically inactive low-molecular-mass fragments.

Amelogenesis imperfecta denotes a group of inherited enamel malformations with various clinical phenotypes and inheritance. In a family carrying the autosomal recessive pigmented hypomaturation type of amelogenesis imperfecta, a mutation in the MMP20 (enamelysin) has been characterized (Kim et al, 2005). This mutation is likely to disrupt the splicing of RNA transcripts causing reduced mRNA levels and loss of the functional hemopexin domain of the protein resulting in the reduction of MMP20 activity.

2.3.3 The knockout mouse models of different MMPs

As described in the previous chapter, all MMPs have multiple substrates including also several other molecules than matrix components. A lot of the substrate specificity data has been obtained from various *in vitro* experiments. However, it is difficult to translate this knowledge to physiologic and pathologic conditions where certain concentration of multiple MMPs and different substrates co-exist at any specific time and site. Since 1990s, several knockout mouse models have been generated making it possible to study *in vivo* functions of different MMPs (Table 2.2). Somewhat surprisingly, most of these loss-of-function mouse models show minimal or no phenotype during normal physiologic conditions. Nevertheless, the challenging of these mouse models for different pathologies has revealed roles for MMPs in various pathologic processes. In several cases, the functions of MMPs are not related to ECM degradation but to regulation of such bioactive molecules as chemokines, cytokines, apoptotic ligands or angiogenic factors. Furthermore, specific phenotypes have been identified in mice with combined deficiency of two different MMPs.

Table 2.2. Knockout mouse models of different MMPs

	Spontaneous phenotype	**Phenotype after pathological challenge**	
		Tumoral	**Non-tumoral**
Mmp1a$^{-/-}$			
Mmp2$^{-/-}$	↓Body size ↓Bone density Altered mammary branching	↓Tumor growth ↓Angiogenesis ↓Acute hepatitis	↑Arthritis ↓Resolution of lung allergic inflammation
Mmp3$^{-/-}$	Altered mammary branching	↑Skin carcinogenesis	↓Contact dermatitis ↓Acute hepatitis ↑Arthritis
Mmp7$^{-/-}$	↑Innate intestinal immunity Defective prostate involution		Impaired tracheal wound repair ↓Pulmonary fibrosis ↓EAE*
Mmp8$^{-/-}$		↑Skin carcinogenesis ↑Experimental metastasis	↑Asthma ↓Acute hepatitis ↓EAE*
Mmp9$^{-/-}$	Delayed growth plate vascularization Defective endochondral ossification Delayed myelinization	↓Experimental metastasis	↓Aortic aneurysms ↓Arthritis ↓Acute hepatitis Prolonged contact dermatitis ↓Colitis
Mmp10$^{-/-}$			↑Pulmonary inflammation and mortality

	Spontaneous phenotype	Phenotype after pathological challenge	
		Tumoral	Non-tumoral
Mmp11$^{-/-}$			Accelerated neointima formation after vessel injury
Mmp12$^{-/-}$	Delayed myelinization		↓Pulmonary emphysema ↑EAE*
Mmp13$^{-/-}$	Bone remodelling defects		↓Hepatic fibrosis
Mmp14$^{-/-}$	Severe abnormalities in bone and connective tissue Defective angiogenesis Premature death		
Mmp16$^{-/-}$	Growth retardation		
Mmp17$^{-/-}$			
Mmp19$^{-/-}$		↓Skin carcinogenesis ↑Angiogenesis	↑Induced obesity
Mmp20$^{-/-}$	Amelogenesis imperfecta		
Mmp24$^{-/-}$			Absence of mechanical allodynia
Mmp28$^{-/-}$			↑Macrophage recruitment in lungs

Based on Fanjul-Fernández et al. (2010) and references therein.
*EAE indicates experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis.

2.4 ADAM (a disintegrin and a metalloproteinase) family of proteinases

ADAMs as well as ADAMTS (ADAMs with thrombospondin motifs) constitute the adamalysin family of metzincins (Bode et al, 1993; Rawlings et al, 2012). ADAMs are anchored to the cell surface through a C-terminal transmembrane domain, while ADAMTS are secreted proteins.

The domain structure of ADAMs typically comprises propeptide, metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor (EGF) -like, transmembrane and cytoplasmic domains. Disintegrin, cysteine-rich and EGF-like domains differentiate them from MT-MMPs. The disintegrin domain can mediate cell-cell and cell-matrix bindings through integrins. Originally, this was suggested to have a role in egg-sperm fusion in mammalians. The cysteine-rich domain is supposed to interact with proteoglycans of the heparan sulphate class. The functions of the EGF-like domain are not known. Through the cytoplasmic domain ADAMs can interact with intracellular signaling proteins and cytoskeletal proteins. The metalloproteinase domain shares considerable similarity with that of MMPs, but only 12 of the known 22 human ADAMs contain an active metalloproteinase domain (Klein & Bischoff, 2011). The metalloproteinase activity of particular ADAMs is inhibited by specific TIMPs as will be described in chapter 2.5.

This section of the review focuses on the 12 ADAMs which contain an active MMP domain and can at least potentially degrade various ECM components and be inhibited by TIMPs. The main *in vivo* functions of ADAMs are, however, related to ectodomain shedding, i.e. proteolytic releasing of membrane-anchored proteins (e.g. growth factors, cytokines, receptors, adhesion molecules) (Edwards et al, 2008). ADAM8 has been linked to inflammatory processes (e.g. asthma, rheumatoid arthritis), but it is not essential for normal development in mice (Kelly et al, 2005). ADAM9 has been linked to poor prognosis in prostate and renal cancers (Klein & Bischoff, 2011). ADAM9 deficient mice are born healthy but develop retinal degeneration during ageing (Parry et al, 2009; Weskamp et al, 2002). ADAM8 and ADAM9 are not inhibited by any of the TIMPs (Table 2.4). ADAM10 is associated with cancer progression and metastasis. In inflammation it can have a stimulatory or reducing effect (Klein & Bischoff, 2011). ADAM10 knockout mice die during embryogenesis exhibiting defects in the development of the central nervous system and the vasculature (Hartmann et al, 2002). ADAM12 is associated to muscular development and degeneration, adipogenesis and tumor progression (Klein & Bischoff, 2011). The disintegrin domain of the ADAM15 contains an RGD motif, which mediates cell-cell and cell-matrix contacts to various partners through integrin-mediated binding. ADAM15 null mice are born healthy but show reduced neovascularization after hypoxia-induced retinopathy (Horiuchi et al, 2003). Among the ADAMs, the best characterized one is ADAM17. It is also known as TNF (tumor necrosis factor) converting enzyme (TACE) as it functions as the major sheddase responsible for releasing soluble TNF from cell surface. ADAM17 also participates in shedding of TNF receptors p55 and p75, L-selectin, E-selectin and epidermal growth factor (EGF) receptor ligands (e.g. EGF, transforming growth factor-α) (Edwards et al, 2008; Klein & Bischoff, 2011). Unlike other TIMPs, TIMP3 is an efficient inhibitor of ADAM17 and in several inflammatory processes (such as liver regeneration, LPS induced septic shock) TIMP3 has an essential role in controlling TNF processing (Mohammed et al, 2004; Smookler et al, 2006). ADAM19 expression has been associated with the invasive primary brain tumors and renal cell carcinoma (Klein & Bischoff, 2011), but in mice it is vital for normal heart development (Zhou et al, 2004). ADAM20 and ADAM30 are expressed in testis, but their biological functions are not known. ADAM21 is expressed in testis and during the development of nervous system (Yang et al, 2005; Yi et al, 2010), but its *in vivo* functions remain to be shown. ADAM28 is expressed in epididymis and in developing tooth, although its significance in these or other tissues is not well characterized (Klein & Bischoff, 2011). ADAM33 is associated with asthma in human populations. Based on expression data, its role in asthma is supposed to be related to remodeling of the airways and not to inflammatory component (Klein & Bischoff, 2011). ADAM33 deficient mice develop normally and react similarly to control animals in an experimental allergen-induced asthma model (Chen et al, 2006).

In contrast to the ADAMs, the members of the ADAMTS protease family are secreted proteins. The ADAMTSs are constituted of propeptide, metalloproteinase and disintegrin domains. The disintegrin domain is linked to a central thrombospondin type I -like repeat, a cysteine-rich domain and varying numbers of C-terminal thrombospondin repeats (Porter

et al, 2005). TIMP3 exhibits the widest inhibitory pattern against ADAMTS, while other TIMPs can inhibit only ADAMTS4, if any (Brew & Nagase, 2010). Important biological functions have been shown for several ADAMTS (Apte, 2009). *In vitro*, ADAMTS2, 3 and 14 have been shown to participate in processing of fibrillar procollagen types I–III, and indeed ADAMTS2 mutations are linked to Ehlers-Danlos syndrome, a connective tissue disorder characterized with impaired collagen assembly. ADAMTS13 contributes to blood coagulation through proteolytic processing of von Willebrand factor. Several ADAMTS are able to degrade large aggregating proteoglycans (e.g. aggrecan, versican and brevican) and might have a role in pathologies such as osteoarthritis.

2.5 Tissue inhibitors of metalloproteinases (TIMPs)

In mammalians, the gene family of tissue inhibitors of metalloproteinases comprises four members that share significant homology in their structure. Originally, TIMPs were considered as interchangeable MMP inhibitors, but more and more data is accumulating describing differences between TIMPs including discovery of novel functions, some of which are not related to inhibition of MMPs.

TIMPs are secreted proteins consisting of ~25 amino acid (aa) signal peptide and ~180–200 aa mature peptide with molecular mass of 21—28 kDa (Table 2.3). Each TIMP has 12 conserved cysteine residues, which form 6 disulphide bridges. The N-terminal domain of each TIMP spans two-thirds of the polypeptide and contains another conserved area, VIRAK-sequence. The N-terminal domain is conserved in all TIMPs and it is responsible for MMP inhibition, while the C-terminal domain contains more variability and plays a role in proMMP binding.

Table 2.3. Molecular characteristics of TIMPs

	TIMP1	**TIMP2**	**TIMP3**	**TIMP4**
Chromosomal locus (human/mouse)	X11p11.23–11.4 / X A1.3	17q23–25 / 11 E2	22q12.1–q13.2 / 10C1-D1	3p25 / 6 E3
Located inside gene	*Synapsin 1*	–	*Synapsin 3*	*Synapsin 2*
mRNA size (kb)	0.9	3.5–1.0	5.0–2.4	1.4
Amino acids in mature peptide (signal peptide)	184 (23)	194 (26)	188 (23)	194 (29)
Molecular mass* (kDa)	20,709	21,755	21,690	22,329
N-glycosylation sites	2	0	1	0
Protein localization	Soluble	Soluble/cell surface	ECM	Soluble
ProMMP association	ProMMP9	ProMMP2	ProMMP2/9	ProMMP2
MMPs poorly inhibited	MMP14, MMP16, MMP19 and MMP24	None	None	None

* indicates human protein without any glycans
Modified from Lambert et al. (2004) and Brew et al. (2010).

The mechanism of MMP inhibition is based on the binding of the wedge shaped N-terminal domain of TIMP to the active site of the MMP (Fernandez-Catalan et al, 1998; Gomis-Rüth et al, 1997). This forms a non-covalent 1:1 stochiometric complex. In general, all TIMPs are potent inhibitors of all MMPs, with the exception that TIMP1 is a poor inhibitor of MT-MMPs and MMP19 (Brew & Nagase, 2010). In addition to inhibition of MMPs, every TIMP is also capable of inhibiting some members of the other metalloproteinase families ADAMs and ADAMTSs. While TIMP1, 2 and 4 inhibit only a few of them, TIMP3 is an efficient inhibitor of many ADAMs (Table 2.4) and ADAMTS1, 2, 4 and 5 (Brew & Nagase, 2010).

Table 2.4. Inhibition of ADAMs by TIMPs

	TIMP1	TIMP2	TIMP3	TIMP4	Reference(s)
ADAM8	(+)	-	-	-	Amour et al, 2002
ADAM9	-	-	-	NA	Amour et al, 2002
ADAM10	+	-	+	-	Amour et al, 2000
ADAM12	-	-/+	+	-	Jacobsen et al, 2008; Loechel et al, 1999; Roy et al, 2004
ADAM15	-	(+)	+	NA	Maretzky et al, 2009
ADAM17	-/(+)	-/+	+	-/+	Amour et al, 1998; Lee et al, 2005a
ADAM19	-	-	-/+	NA	Chesneau et al, 2003; Wei et al, 2001
ADAM20					
ADAM21					
ADAM28	-	-	+	+	Howard et al, 2001; Mochizuki et al, 2004
ADAM30					
ADAM33	-	(+)	+	+	Zou et al, 2004

Symbols - and + indicate no inhibition and inhibition, respectively. If in parentheses, inhibition was achieved only with a very high concentration, which is unlikely to have a role *in vivo*.

Evolutionally the *Timp* gene family appears conserved. In mammals, the family is composed of four members and in Drosophila of one gene (Pohar et al, 1999). Drosophila *Timp* participates in wing maturation and its null mutation results in premature death of flies with inflated wings, bloated guts and tissue autolysis (Godenschwege et al, 2000; Kiger et al, 2007). As will be discussed later, none of the *Timp* knockout mice exhibit a similar severe phenotype, but the effects of combined deficiencies are still unknown.

2.5.1 Biology of TIMP1

TIMP protein was first purified from human fibroblast cultures (Vater et al, 1979; Welgus et al, 1979). In 1985, the human *TIMP1* cDNA was cloned and it was found to be identical to erythroid-potentiating activity (EPA), a protein that was purified and cloned earlier and shown to be able to stimulate cells of the erythroid lineage (Docherty et al; Gasson et al). It is widely expressed in various embryonic and adult tissues (Nuttall et al, 2004). In cell culture, TIMP1 expression can be stimulated by various growth factors and cytokines (Lambert et al, 2004).

Unlike other TIMPs, TIMP1 does not inhibit MT-MMPs (Table 2.3). In addition to EPA, TIMP1 has growth promoting effects on various cell types *in vitro* (Brew & Nagase, 2010). This activity has been characterized to be unrelated to MMP inhibition. The growth promoting activities of TIMP1 and TIMP2 have been associated to Ras activation, through the tyrosine kinase / mitogen activated protein kinase (MAPK) for TIMP1 and through protein kinase A activation for TIMP2 (Wang et al, 2002). TIMP1 has also been shown to suppress growth or to have an anti-apoptotic effect on some cell lines (Brew & Nagase, 2010). On the cell surface, TIMP1 has been shown to interact with CD63 and integrin β1, and may thus contribute to regulation of cell polarity and apoptosis (Jung et al, 2006). Like other TIMPs, TIMP1 has been shown to have antiangiogenic effects (Handsley & Edwards, 2005).

Generation of *Timp1* knockout mice has greatly increased knowledge of *in vivo* functions of TIMP1 (Soloway et al, 1996). These mice are born healthy and were originally characterized with a quite mild phenotype: female mice have reduced length of estrus cycle and reduced reproductive life span (Nothnick, 2000; Nothnick, 2001); and at the age of 4 months, unfavorable changes in heart geometry could be detected (Roten et al, 2000). Later, impaired learning and memory processes have been characterized in *Timp1* null mice (Chaillan et al, 2006; Jourquin et al, 2005). Subjecting these mice to various experimental challenges has revealed roles for TIMP1 in multiple other systems. In liver regeneration after partial hepatectomy, TIMP1 seems to negatively regulate hepatocyte growth factor (HGF) activity by regulating MMP mediated activation and release of pro-HGF from the ECM (Mohammed et al, 2005). *Timp1* deficiency protects mice from *Pseudomonas aeruginosa* corneal and pulmonary infections through MMP-dependent mechanism (Lee et al, 2005b).

2.5.2 Biology of TIMP2

In 1989, Goldberg et al. and Stetler-Stevenson et al. found a new protein, which formed a 1:1 complex with MMP2. The amino acid sequence of the protein resembled that of TIMP1 and it was able to inhibit MMP2 activity and was thus named TIMP2. *Timp2* is expressed in most tissues, and in contrast to *Timp1* its expression is more constant and not stimulated by growth factors and cytokines *in vitro* (Lambert et al, 2004; Nuttall et al, 2004).

TIMP2 is an efficient inhibitor of both soluble and MT-MMPs. Biochemical studies of TIMP2 have revealed a novel and unexpected function for TIMPs, i.e. interaction with proMMPs (Table 2.3). The best characterized example of this function is the activation of proMMP2 in a process involving also TIMP2 and two MT1-MMPs (Brew & Nagase, 2010; Strongin et al, 1995). The hemopexin domain of proMMP2 and the C-terminal domain of TIMP2 bind together. The TIMP2 of this complex binds through its N-terminal domain to the catalytic site of the MT1-MMP. When this trimolecular complex in formed, the activation of proMMP2 is performed through a proteolytic cleavage by another MT1-MMP. Formation of this complex localizes the activation of MMP2 on the cell surface, where proteolytic activity is needed in processes such as cell migration. Other TIMPs are also able to interact with proMMP2 or 9 (Table 2.3), but the *in vivo* functions of these interactions are not known.

Similar to TIMP1, TIMP2 possesses eryhroid-potentiating activity and growth promoting activity in many cell lines (Hayakawa et al, 1994; Stetler-Stevenson et al, 1992). TIMP2 has an antiangiogenic effect, which is reported to be unrelated to MMP inhibition and mediated through interaction of TIMP2 and integrin α3β1 (Seo et al, 2003). In addition, antiangiogenic effects are likely mediated via MMP inhibition by TIMP2 and RECK. TIMP2 has been shown to increase the expression of RECK, which can inhibit MMP2, 9 and 14 and ADAM10 (Oh et al, 2004a). Studies of the possible role of TIMP2 in apoptosis have been controversial (Brew & Nagase, 2010).

Using gene targeting techniques, two different *Timp2* deficient mouse models have been produced. One lacks exons 2 and 3 and the other lacks the first exon and ~3kb of the promoter sequences of *Timp2* (Caterina et al, 2000; Wang et al, 2000). The previous one was reported to produce a truncated mRNA and a corresponding protein, which, however, lacks the MMP inhibitory activity. Although originally reported as a complete null mutation, also the latter one was recently shown to produce a truncated TIMP2, which does not inhibit MMPs (Jaworski et al, 2007). Originally, in the both models activation of proMMP2 was shown to be reduced, but these mice develop, grow and reproduce normally. Several years later, other phenotypes were characterized relating to central nervous system (abnormal behavior and motor functions) and myogenesis (Jaworski et al, 2005; Jaworski et al, 2006; Lluri et al, 2006; Lluri et al, 2008)

The genomic locus of *Timp2* differs from that of other *Timp*s, which are located within *Synapsin* genes. In mouse or human genome there are no *Synapsin* genes in the same chromosome as *Timp2*. Moreover, *Timp2* contains another gene, *Ddc8*, within its first intron. In contrast to *Synapsin-Timp* loci, *Timp2* and *Ddc8* are located in the same orientation. Interestingly, Jaworski et al. (2007) reported that in brain, portion of *Timp2* mRNA contains sequence for *Ddc8* likely through alternative splicing. In addition, the expression of *Ddc8* mimics the expression pattern of *Timp2*.

2.5.3 Biology of TIMP3

TIMP3 was first found as an ECM component, which was transiently expressed during oncogenic transformation of chicken embryo fibroblasts (Pavloff et al, 1992; Staskus et al, 1991). It was named TIMP3, because its amino acid sequence resembled other TIMPs and it possessed inhibitory activity to MMPs. In terms of structure and metalloproteinase inhibitory profile, human TIMP3 is evolutionally the most conserved TIMP as it is the closest relative to the single TIMP in Drosophila (Pohar et al, 1999; Wei et al, 2003). TIMP3 is an efficient inhibitor of MMPs including MT-MMPs. Similarly to TIMPs 1 and 2, it is widely expressed in embryonic and adult tissues (Nuttall et al, 2004).

TIMP3 has several features that distinguish it from the other TIMPs. First, it is tightly bound to the ECM. In contrast to other TIMPs, the N-terminal domain of TIMP3 contains an excess of positive charges, which are likely to be responsible for its binding to ECM molecules, such as heparan sulphate and other sulphated glycosaminoglycans (Yu et

al, 2000). Secondly, it inhibits a wide variety of members of ADAM and ADAMTS families (Table 2.4). Through the inhibition of these proteases, TIMP3 might have a more important role in coordinating of shedding of several membrane-bound growth factors, receptors and other bioactive molecules. Thirdly, TIMP3 is the only TIMP that has been linked to a human disease. Mutations of *TIMP3* result in deposition of excessive amounts of TIMP3 in Bruch's membrane of the retina causing blindness at an early age (Li et al, 2005). The disease is known as Sorby's fundus dystrophy (Box 2.2).

Box 2.2. Mutant *TIMP3* is related to Sorby's fundus dystrophy, a macular degenerative disease in humans

Mutations in exon 5 of the *TIMP3* gene have been linked to Sorby's fundus dystrophy (SFD), a rare inherited disease causing blindness (Li et al, 2005). Clinically and histopathologically it resembles the exudative form of age-related macular degeneration (AMD), which is the most common cause of blindness in the elderly of developed countries, but exhibits an earlier age of onset. Thus, characterization of SFD pathogenesis may provide essential information for prevention and therapy of AMD.

SFD is an autosomal dominant disorder which typically manifests at the beginning of the fifth decade. In a majority of cases, the patient loses central vision as a result of neovascular macular degeneration. Aggregates of TIMP3 have been detected in Bruch's membrane of SFD patients.

In exon 5 of *TIMP3* several independent mutations have been discovered resulting in SFD. Most of the mutations result in unpaired number of cysteine residues in the C-terminal domain. Mutant TIMP3 proteins can form dimers and maintain MMP inhibitory acitivity, but their functions are still poorly understood. A knock-in mouse model with one of the typical human mutations (Ser156Cys) exhibits a phenotype similar to early stages of the human disease (Weber et al, 2002).

In different cancer cell lines, TIMP3 has been shown to induce apoptosis (Brew & Nagase, 2010). In melanoma cells, this is mediated through stabilization of certain death receptors (TNF receptor 1, FAS and TNF-related apoptosis inducing ligand receptor 1) (Ahonen et al, 2003). TIMP3 also possesses an antiangiogenic effect. Other than metalloproteinase inhibitory activity has been characterized to mediate this effect, i.e. blocking of vascular endothelial growth factor (VEGF) binding to VEGF receptor 2 and through TIMP3 interaction with angiotensin II type 2 receptor (Kang et al, 2008; Qi et al, 2003). Although TIMP3 is recognized as the ECM-bound TIMP, it has been shown that synthesized TIMP3 can also be endocytozed by a scavenger receptor LRP (low-density lipoprotein related protein) in osteosarcoma cell line (Troeberg et al, 2008).

Timp3 knockout mice were first found to have accelerated apoptosis during mammary gland involution and to develop spontaneous air space enlargement in the lungs (Fata et al, 2001; Leco et al, 2001). Later, several other spontaneous phenotypes have been characterized. At the age of 21 months, *Timp3* knockout mice develop heart failure, which resembles human dilated cardiomyopathy (Fedak et al, 2004). Aging of *Timp3* deficient mice results also in cartilage degradation in hind joints resembling osteoarthritis in patients (Sahebjam et al, 2007). By inhibiting ADAM17, i.e. TACE, which controls

processing of TNF, TIMP3 appears to possess an important regulatory role in various inflammatory processes. This was demonstrated by impaired liver regeneration and increased mortality in response to lipopolysaccaride (LPS) shock in *Timp3* deficient mice (Mohammed et al, 2004; Smookler et al, 2006).

2.5.4 Biology of TIMP4

The most recent member of the *Timp* gene family, *Timp4*, was discovered by searching ESTs (expressed sequence tags) containing homologous sequences with known *Timp* genes. Consequently, full-length cDNA sequence was cloned from a cDNA library derived from human heart (Greene et al, 1996). TIMP4 protein contains 12 cysteine residues and a consensus sequence (VIRAK) in the N-terminal domain, two features that all mammalian TIMPs share (Figure 2.1). Comparison of murine TIMP amino acid sequences revealed that TIMP4 resembles more TIMP2 and TIMP3 than TIMP1 (Leco et al, 1997). Cloning of human *TIMP4* gene confirmed that it contains five small exons like other *TIMPs* (Olson et al, 1998). Cloning and characterization of the murine *Timp4* gene in this study coincided with a similar study by Young et al. (2002) as will be discussed under Results and Discussion.

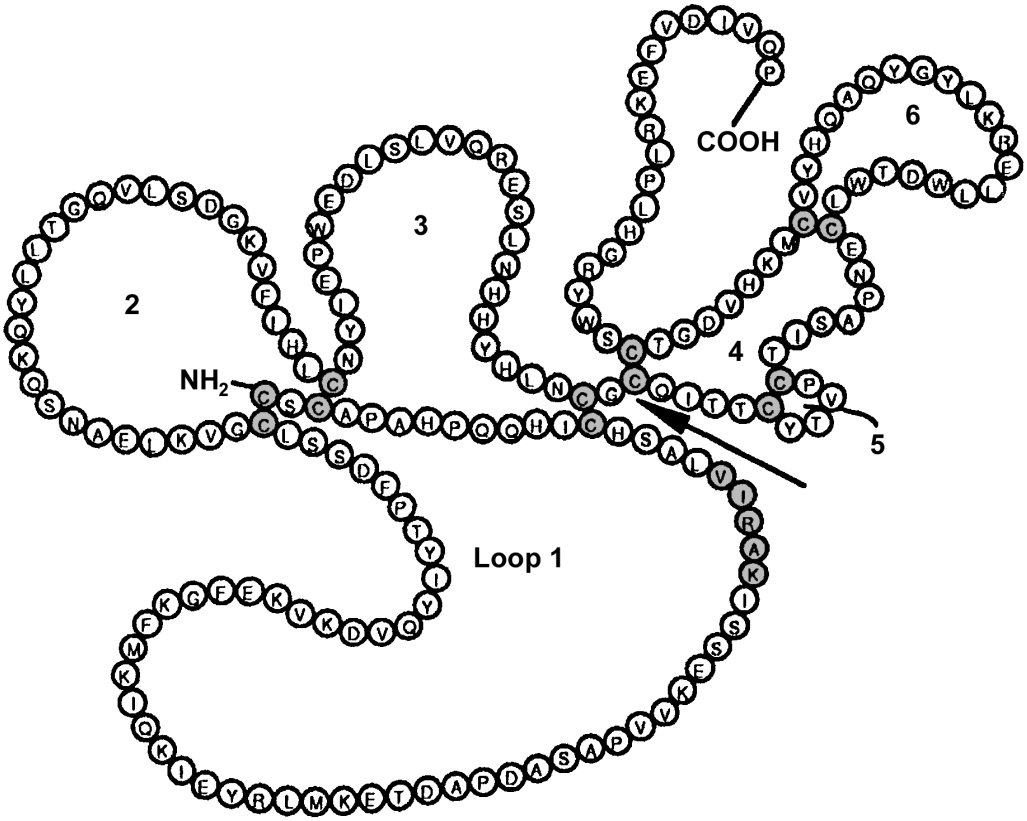

Figure 2.1. Amino acid sequence and loop structure of human TIMP4 protein. The 12 cysteines forming the disulfide bonds and the conserved VIRAK sequence are shaded. Arrow indicates the boundary between N- and C-terminus. Modified from Douglas et al. (1997).

All analyses of tissue distribution of TIMP4 are based on the measurement of the corresponding mRNA levels in tissues or RNA preparations extracted from murine and human tissues. Such studies will be presented and discussed in conjunction of the results of the present study under Results and Discussion.

Biological activity of TIMP4

To date, several studies have shown that TIMP4 is a general MMP inhibitor and is able to inhibit at least some members of each MMP subgroup including MT-MMPs (Table 2.5). Literature suggests a special role for the interactions of TIMP4 and some specific MMPs. First, TIMP4 has the ability to bind to the hemopexin domain of proMMP2 in a similar manner as TIMP2 during the activation process of proMMP2 in trimolecular complex of MT1-MMP, proMMP2 and TIMP2 (Bigg et al, 1997). However, TIMP4 cannot substitute TIMP2 in this process and activate proMMP2 (Bigg et al, 2001). In contrast, TIMP4 inhibits proMMP2 activation through MT1-MMP inhibition (Groft et al, 2001). Moreover, proMMP2 activation is not altered in *Timp4* deficient mouse embryonic fibroblasts (English et al, 2006). Thus, the function of TIMP4 binding to proMMP2 remains unknown. Secondly, among several MMPs (types 1, 2, 3, 7, 9 and 26), TIMP4 is the most potent inhibitor of MMP26 (Liu et al, 1997; Zhang et al, 2002), an enzyme that is also capable of activating proMMP9 (Uría & López-Otín, 2000). In endometrial tissue, the cyclic expression pattern of *Timp4* mRNA resembles that of MMP26 (Pilka et al, 2004). Moreover, MMP26 and TIMP4 staining correlates with the depth of myometrial invasion in endometrial cancer (Tunuguntla et al, 2003), and TIMP4 is co-localized with MMP26 in human breast ductal carcinoma *in situ* (Zhao et al, 2004b). TIMP4 blocks MMP26 mediated proMMP9 activation *in vitro* (Zhao et al, 2004b), but the significance of MMP26/TIMP4 coupling remains to be clarified *in vivo*.

Table 2.5. Matrix metalloproteinases inhibited by TIMP4

	Reference(s)
MMP1, 2, 3, 7, 9	Liu et al, 1997
MMP19	Stracke et al, 2000b
MMP26	Zhang et al, 2002
MMP8, 9, 12, 13, MT1-MMP*	Stratmann et al, 2001
MMP2, MT1-MMP	Bigg et al, 2001
MT1-MMP	Hernandez-Barrantes et al, 2001
MT2-MMP	Morrison et al, 2001
MMP1, 2, 3, MT1-MMP	Troeberg et al, 2002
MTI1-MMP, MT3-MMP	Zhao et al, 2004a

*studied using recombinant N-terminal domain of TIMP4

In contrast to MMPs, only a few of ADAMs and maybe none of the ADAMTSs can be inhibited by TIMP4 (Melendez-Zajgla et al, 2008). Among ADAM/TIMP interactions, especially interesting and biologically significant is the efficient inhibition of ADAM17

by TIMP3 (Amour et al, 1998) that orchestrates a wide spectrum of biological processes by controlling the shedding of TNF and several other membrane-bound proteins (Kassiri et al, 2005; Mohammed et al, 2004; Smookler et al, 2006). Lee et al. (2005a) have shown that removal of the C-terminal domain makes ADAM17/TIMP4 binding possible, but only after further genetic modification of the N-terminal domain, the mutant TIMP4 protein becomes an efficient inhibitor of ADAM17. Thus, ADAM/TIMP-specificity is dependent on both N- and C-terminal domains of TIMP.

A number of studies have linked TIMP4 to regulation of proliferation and apoptosis. Tummalapalli et al. (2001) demonstrated that purified TIMP4 induces apoptosis in human cardiac fibroblasts transformed with polyoma virus middle T-antigen, but not in normal cardiac fibroblasts. Lovelock et al. (2005) reported that adenovirus-mediated TIMP4 overexpression, but not a broad-spectrum MMP inhibitor RS-130830, stimulated cardiac fibroblast proliferation. Apoptosis was not affected by TIMP 4 in this study. In rat smooth muscle cells, adenovirus-mediated TIMP4 overexpression induced apoptosis *in vitro* and *in vivo* (Guo et al, 2004). In contrast, TIMP4 inhibits apoptosis in human breast cancer cells *in vitro* and in mammary tumor xenografts *in vivo* (Jiang et al, 2001). Nevertheless, TIMP4 inhibits growth of Wilms' tumor cells *in vitro* and *in vivo* (Celiker et al, 2001). Thus, effects of TIMP4 on apoptosis and cell growth are opposing in different cell types. The underlying mechanisms are poorly known, but also MMP-independent mechanisms have been suggested. Jung et al (2006) showed that interaction of TIMP1 with CD63 and integrin β1 regulates apoptosis. TIMP4/CD63 interaction has been reported, but biological effects of this interaction have not been displayed (Chirco et al, 2006).

During the past decade it has became obvious that *in vivo* functions of many MMPs are related to inflammation and not only to ECM remodeling (Parks et al, 2004). Through controlling of MMPs and to some extent of ADAMs/ADAMTS, the role of TIMPs in inflammation is expected to be at least as important. The more restricted expression of TIMP4 in the cardiovascular system also justified a detailed evaluation of TIMP4 in the context of cardiovascular pathologies with inflammatory component.

2.6 TIMP4 in animal models exhibiting cardiovascular phenotypes

To study the pathogenesis of cardiovascular diseases, various genetic, pharmacologic and surgical challenges of laboratory animals have been used. These models have produced significant information of the involvement of TIMP4 in cardiovascular pathologies.

Timp4 as well as the other *Timps* are expressed in normal murine heart (Nuttall et al, 2004). Ischemia-reperfusion injury results in release of TIMP4 from the myocardium into the coronary effluent (Schulze et al, 2003). Decreased TIMP4 levels within and around infarcted myocardium have been reported in rodents and larger animals after induction of infarction using surgical ligation of coronary artery (Mukherjee et al, 2003; Peterson et al, 2000; Vanhoutte et al, 2006; Wilson et al, 2003). Concerning the dynamic

role of the healing of myocardial infarction (Vanhoutte et al, 2006), interpretation of the biologic role of TIMP4 is challenging. During early healing of infarcted myocardium, various inflammatory cells migrate to the infarct tissue and extensive proteolysis by MMPs is present. Next, the inflammation is gradually replaced by new ECM and blood vessels during scar formation. Thereafter, remodeling of the myocardium continues for months after infarction. In all these processes TIMP4 could be involved. Moreover, TIMP4 is involved in biologic processes preceding myocardial infarction: TIMP4 is present throughout the arterial wall after vascular injury (Dollery et al, 1999); and TIMP4 is the major TIMP in human platelets regulating platelet aggrecation and recruitment (Radomski et al, 2002).

In studies of heart failure various approaches have been used. In general, heart is challenged by pressure or volume overload or some other methods inducing cardiomyopathies. Despite differences in model systems, literature suggests that TIMP4 is generally decreased in heart failure. TIMP4 is decreased in spontaneously hypertensive heart failure rats (Li et al, 2000) as well as in heart failure induced by myocardial infarction (Peterson et al, 2001; Peterson et al, 2000; Seeland et al, 2002). Moreover, TIMP4 is decreased in diastolic and systolic atrial failure induced by rapid atrial pacing (Hoit et al, 2002).

2.7 TIMP4 in human cardiovascular diseases

In the context of human cardiovascular system, studies focusing on the role of any specific gene/protein system in certain pathology are complicated. The pathologic processes are dynamic and may develop over several years. Furthermore, analysis of the results is complicated by various genetic and environmental backgrounds. So far, a majority of the human-based studies concerning TIMP4 in cardiovascular diseases have been descriptive and have used myocardial samples collected at the end of a pathologic process, e.g. at the time of heart transplantation. Nevertheless, in accordance with animal studies, decreased TIMP4 levels are a common finding in different types of myocardial pathologies as discussed below.

In the human heart, TIMP4 is present in all four chambers (Mukherjee et al, 2006). Li et al. (1998) have shown that TIMP4 is decreased in ischemic cardiomyopathy but not in idiopathic dilated cardiomyopathy. However, patients with deteriorating dilated cardiomyopathy have decreased cardiac TIMP4 levels in comparison to patients with stable disease (Felkin et al, 2006). In cardiac surgery, myocardial TIMP4 is decreased during cardiopulmonary bypass suggesting that decreased TIMP4 levels are not just a hallmark of end-stage heart disease, but regulation of TIMP4 is involved also in acute pathologic processes of the heart (Mayers et al, 2001).

When this investigation was started, no data was available on the involvement of TIMP4 in human vascular pathologies. Therefore, new findings on the potential role of TIMP4 in vascular diseases will be discussed in the context of the present results below.

3. AIMS OF THE PRESENT STUDY

The purpose of the present study was to clarify the role of TIMP4 in the regulation of ECM turnover with special reference to the cardiovascular system. It was hypothesized that TIMP4 is an essential regulator of connective tissue turnover in cardiovascular structures. To test this hypothesis the following specific aims were set for this study:

1. To clone and characterize the murine *Timp4* gene and to resolve its temporo-spatial expression pattern.

2. To produce genetically modified mice lacking *Timp4* gene and to characterize their phenotype.

3. To investigate the function of TIMP4 in cardiovascular diseases by subjecting *Timp4* deficient mice to different experimental models of cardiovascular diseases.

4. To test the consequences of *Timp4* deficiency in processes where the role of TIMPs and MMPs is well established, i.e. dermal wound healing, tumorigenesis and metastasis.

5. To evaluate the role of TIMP4 in human cardiovascular diseases by determining TIMP4 expression and localization in vascular wall samples from patients with inflammatory cardiovascular disorders.

4. MATERIALS AND METHODS

4.1 Biological material

4.1.1 Experimental animals (*II*, *III*)

The generation of knockout mice and the procedures used for primary phenotypic and dermal wound healing characterization were approved by the Institutional Committee for Animal Welfare, University of Turku, Turku, Finland. Animal protocols for studies of angiogenesis, tumorigenesis, metastasis, cardiac pressure overload and myocardial infarction were approved by the Ontario Cancer Institute Animal Care Committee, Toronto, Canada in accordance with guidelines of the Canadian Council for Animal Care.

4.1.2 Human samples (*IV*)

Atherosclerotic coronary artery samples were collected from patients at the time of cardiac transplantation at the Helsinki University Central Hospital (HUCH), Helsinki, Finland. Non-atherosclerotic coronary artery samples were obtained from healthy organ donors without history of cardiovascular disease, who had been excluded from transplantation due to tissue type or size mismatch. Heart endomyocardial biopsy (EMB) samples were collected from right ventricles of cardiac allograft recipients at the time of routine rejection surveillance examinations. Institutional ethical review board of the HUCH approved the study protocol, and informed consent was obtained from all patients.

Temporal artery biopsies were performed for patients that were under clinical examination due to high blood sedimentation rate. Samples fulfilling the criteria of giant cell arteritis (GCA) (Hunder et al, 1990) were used as arteritis specimens while samples without any signs of inflammation served as controls.

4.2 Methods

4.2.1 Cloning of murine *Timp4* gene (*I*)

As the first step of cloning experiments, murine *Timp4* cDNA was cloned from total heart RNA using RT-PCR method. Primers were designed based on the published cDNA sequence (Leco et al, 1997). The amplified fragment was cloned into pGEM-T vector (Promega), named pMTIMP4-1 and sequenced.

To isolate murine *Timp4* gene, two mouse genomic libraries, one in LambdaFish II phage (Stratagene, La Jolla, CA, USA) and the other one in peloBAC (Genome Systems, St. Louis, MO, USA) were screened. First, an aliquot of 5 x 10^5 plaques of the LambdaFish II phage library was plated and transferred to Hybond-N nylon membranes (Amersham, Buckinghamshire, England). After standard prehybridization, the filters were hybridized with *Timp4* cDNA probe labelled with [α-^{32}P]dCTP by the random priming method (Boehringer Mannheim, Germany), washed and subjected to autoradiography. The mouse peloBAC library was screened on filters containing pools of genomic clones as recommended by the supplier. Five positive plaques were purified and analyzed using restriction mapping and Southern hybridization. Appropriate fragments were subcloned into pBluescript SK$^+$ vector (Stratagene) and sequenced. The sequence data was analyzed using NIH software BLAST (Basic Local Aligment Search Tool, available at http://www.ncbi.nlm.nih.gov/BLAST/). To search for consensus sequences for putative transcription factor binding sites, TF-Search software (University of Kyoto, Kyoto, Japan) was used.

To discover the 5'-end of mouse *Timp4* cDNA, primer extension analysis was performed as recommended by the supplier (Promega). BLAST software was used for homology search of mouse EST sequences for the 5'-end of *Timp4* transcripts. As another approach for determining 5'-end of *Timp4* gene, 5'-RACE (Rapid amplification of cDNA ends) reaction was performed as recommended by the supplier. Briefly, cDNA synthesis by AMV-reverse transcriptase was performed using a specific oligonucleotide MUSTIMP4AS (5'-CTAGGGCTGGAATGATGT-3') as the primer and total RNA form adult mouse heart as the template. Next, a homopolymeric A-tail was added to the 3'-end of the first strand cDNA by terminal transferase and the cDNA was amplified by PCR using Timp-4-R1 (5'-TGGTTGATTTCATACCGGA-3') and oligo(dT) primers. A second round of PCR amplicifation was performed using T4-prom1 (5'-ATGACACTGCAGAGCCCCA-3') and oligo(dT) as primers and the first round PCR product as a template. The PCR products were cloned into pGEM-T vector (Promega) and sequenced.

4.2.2 Production of *Timp4* knockout mouse (*II*)

To produce *Timp4* knockout mice, a knockout construct, pT4KO, was generated where a 2.4 kb genomic fragment containing exons 1 to 3 of the *Timp4* gene was replaced by 1.6 kb neomycin resistance gene (Figure 4.1.). The 5'-arm (2.6 kb) of the construct was released from T4EX-clone using restriction enzymes BglII and HindIII. The 3'-arm (2.2 kb) resulted from digestion of T4-Sac-clone with restriction enzymes EcoRI and Acc65I. The construct was made by cloning the 5'- and 3'-arms, the neomycin resistance gene (from vector pKO SelectNEO, Stratagene) and the thymidine kinase (from vector pKO Select TK, Stratagene) gene into the multicloning sites of a pKO Scrambler 921 vector (Stratagene).

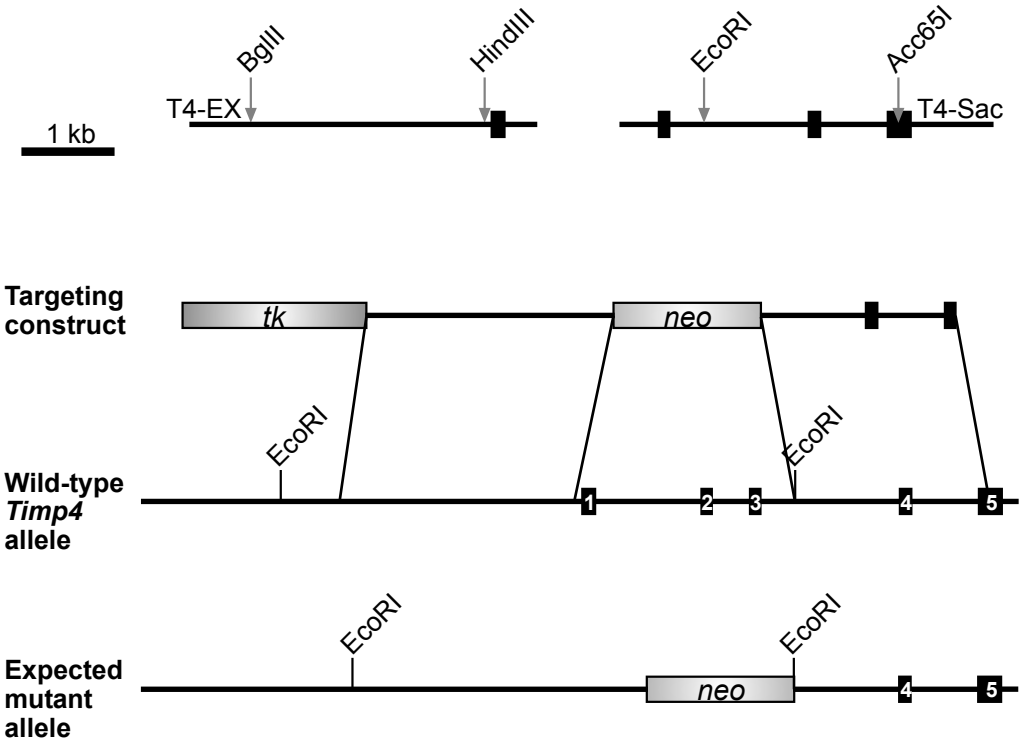

Figure 4.1. Targeting strategy for murine *Timp4* gene. 5'- and 3'-arms are released from genomic clones T4-EX and T4-Sac, respectively. Expected mutant allele is lacking first three exons of *Timp4*. Southern blotting of EcoRI digested genomic DNA results in 0.8 kb shorter fragment in mutant allele. *tk* and *neo* indicate tymidine kinase cassette and neomycin resistance gene, respectively.

The mouse ES cell culture was performed as described earlier (Hogan et al, 1994; Talts et al, 1999). Briefly, mouse ES cells (AB2.2 Prime, Lexicon Genetics Inc) were cultured on irradiated mouse embryonic fibroblasts under conditions suggested by the supplier. 20 μg of linearized pT4KO construct was electroporated into ten million ES cells. Next, the cells were cultured in the presence of selection substrates [300 μg/ml G418 (Gibco) and 2 μM gangiclovir (F. Hoffman-La Roche Inc.)]. The surviving clones were freezed and screened by Southern blotting of EcoRI digested genomic DNA using an external probe pT4KO-5'PCR (*II*). One ES cell clone with the expected mutant *Timp4* locus was expanded and it was injected into C57Bl/6J blastocysts by a commercial service (University of Umeå, Umeå, Sweden). Resulting male chimeras were mated with C57Bl/6J females. The mouseline was named T4KO.

The genotyping of *Timp4* knockout mice was performed either with Southern blotting of EcoRI digested genomic DNA using an external probe (*II*) or with PCR amplification of genomic DNA. Annealing temperature of 58°C was used in standard PCR protocol. For amplification of control and mutated alleles, primers T4-Anc (5'-ACT TCC CAA CTG GGT TTG TTG GTC TGG TCA GA-3') and T4-WT (5'-CCC GTC CAG GGG GCC

ACA GCA AA-3') or primers T4-Anc and T4-neo (5'-TCC TCG TGC TTT ACG GTA TC-3') were used, respectively. PCR reactions resulted in amplification products of 300 and 470 bp for normal and mutated *Timp4* alleles, respectively.

4.2.3 Phenotypic analyses of *Timp4* knockout mouse

4.2.3.1 Pressure overload of heart (II)

Male *Timp4*$^{-/-}$ and control mice were subjected to cardiac pressure overload at the age of 8 weeks by performing constriction of descending aorta (Kassiri et al, 2005). Mice were anesthetized with Ketamine-Xylazine, intubated and ventilated with rodent ventilator. Chest was opened, descending aorta was identified, and ligation was tied around descending aorta and a 27G needle placed parallel to it. Then, the needle was removed, but the ligation was left to produce a constant and permanent constriction of the aorta. In sham operation, ligation of aorta was not tightened and the ligature was not left in thorax. After the closure of the thorax, mice were monitored up to 12 weeks. Cardiac function was monitored by echocardiography at 1, 3, 6 and 12 weeks after operation.

4.2.3.2 Myocardial infarction (II)

To induce myocardial infarction, 10–12 weeks old male *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice were subjected to LAD (left anterior descending coronary artery) ligation as described earlier (Ohta et al, 2004). Mice were anesthetized with Ketamine-Xylazine, intubated and connected to rodent ventilator. Left coronary artery was exposed by opening the thorax and the pericardium, and LAD was ligated. Occlusion of artery was verified by observing the respective area of left ventricle to turn pale indicating ischemia. Next, the thorax was closed and following recovery from anaesthesia, mice were monitored daily. Heart function was evaluated by echocardiography and *in vivo* hemodynamics before sacrifice for tissue collection at 7 days post operation (Kassiri et al, 2005). Sham group was operated similarly except for the ligation of LAD.

4.2.3.3 Wound healing (III)

To study dermal wound healing, two full-thickness circular excisional wounds (diameter 5 mm) were made on the dorsal skin of 7 month old female *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. Wounds were harvested after 3, 5, 7 or 9 days, fixed in formalin and processed for histology. Wound healing was evaluated as percent re-epithelialization [100 x (wound diameter-epidermal gap)/wound diameter] (Escámez et al, 2004). To detect capillary vessels in the healing tissue, immunohistochemical staining for factor VIII related antigen was performed (Cruz-Munoz et al, 2006). Neutrophil infiltration was evaluated after immunostaining using rat anti-neutrophil antibody (Serotec).

4.2.3.4 Tumorigenesis (III)

B16 melanoma cells were used to study tumorigenesis in the absence of *Timp4*. Subcutaneous tumors were generated in *Timp4*$^{-/-}$ and *Timp4*$^{+/+}$ mice by injecting subcutaneously with 1 x 10^5 B16F10 cells. Tumor sizes were measured 5, 7, 9, 11, 14 and 17 days after injection. At 17 days after injection, mice were sacrificed and tumors were fixed in formalin and processed for histology.

In skeletal muscle, tumorigenesis was studied by injecting 7.5 x 10^4 B16F1 melanoma cells intramuscularly into the left hind limb of *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. Tumor growth was assessed by leg diameter measurements every two days. At 14 days after injection, mice were sacrificed and tumors collected to process for histology.

4.2.3.5 Angiogenesis (III)

Angiogenesis was studied *in vivo* as described earlier (McCarty et al, 2002). Briefly, Gelfoam-sponge pieces containing 200 ng of FGF2 (fibroblast growth factor 2) as an angiogenesis stimulus were implanted subcutaneously on both sides of the chest of *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. After 14 days, the mice were sacrificed and the plugs were removed and frozen in OCT compound (Sakura Finetek, Torrance, CA). To measure angiogenesis, endothelial cell migration was assessed by histomorphometric analysis of CD31 immunopositive cells present in the 5 μm thick frozen sections of Gelfoam.

4.2.3.6 Metastasis (III)

To determine the effects of the lack of *Timp4* on pulmonary metastasis, B16F10 melanoma cells (1.2 x 10^5 cells) were injected into the tail vein of *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. At 14 days post-injection, the mice were sacrificed, and the lungs were perfused and fixed with formalin. The amount of tumor colonies on lung surface was counted using stereomicroscopy. The fixed lungs were embedded in paraffin and cut into 5 μm sections. The tumor content was measured by histomorphometry on haematoxylin-eosin stained sections. Angiogenesis was evaluated by counting capillaries after immunostaining for factor VIII related antigen. The proliferating and apoptotic cells were counted after immunostaining for Ki67 positive cells or TUNEL (terminal deoxynucleotidyltransferase-mediated nick end labeling) staining, respectively.

To further dissect the metastasis process, a lung-trap assay was performed as described earlier (Zhang & Hill, 2004). *Timp4*$^{-/-}$ and control mice were intravenously injected with B16F10 cells. Mice were sacrificed at 15 min, 48 h, 96 h and 8 days post-injections and lungs collected. Lung tissues were prepared for a single cell suspension and plated to assess plating efficiency. Part of lung tissue was prepared for lysates by sonication in RIPA buffer and thereafter analyzed by gelatine zymography (Mohammed et al, 2004).

4.2.4 Analytical methods

Analytical methods used in this thesis are summarized in table 4.1. The techniques are described in detail in the original communications as indicated in the table. The cDNA clones used as probes in Northern blot analyses and *in situ* hybridizations are listed in table 4.2 and described in detail in the original publications.

Table 4.1 Analytical methods

Method	Used in Communication(s)
RNA extraction	I and II
Northern hybridization	I and II
Southern hybridization	I and II
RNase protection assay	I
In situ hybridization	I
Taqman RT-PCR	II
Histology and light microscopy	I–IV
In vivo echocardiography	II
Hydroxyproline assay	II
In vivo hemodynamics	II
Gelatine zymography	III
Immunohistochemistry	III and IV
Western blot analysis	IV
Isolation and culture of human inflammatory cells	IV

Table 4.2 cDNA clones used as hybridization probes

mRNA	Clone	Used in Communication(s)	References
Timp1	pMTIMP-1	II	Joronen et al, 2000
Timp2	pMTIMP-2	II	Joronen et al, 2000
Timp3	pMTIMP-3	II	Joronen et al, 2000
Timp4	pMTIMP-4	I and II	I
28S rRNA	pM28S	I and II	Iruela-Arispe et al, 1991

4.2.5 Statistical analyses

Statistical analyses were performed using GraphPad PRISM 3.0 and Excel (Microsoft Corporation). All data are expressed as the mean ± SEM. Data were analyzed by unpaired Student's t-test or by Mann Whitney U-test. P-values less than 0.05 were considered statistically significant.

5. RESULTS AND DISCUSSION

5.1 Murine *Timp4* gene structure (*I*)

In order to produce and investigate a *Timp4* deficient mouse model, the first step of this thesis was to clone and characterize the murine *Timp4* gene. At the time when the cloning experiments were started, the cDNA structure for human *TIMP4* had been published (Greene et al, 1996), and soon also partial human *TIMP4* gene and murine cDNA sequences became available (Leco et al, 1997; Olson et al, 1998).

Initially, murine *Timp4* cDNA was cloned to make screening of genomic clones for *Timp4* possible. Using the published human *TIMP4* cDNA sequence, primers were designed to amplify murine *Timp4* from cDNA made for heart RNA. A 632-bp cDNA clone (pMTIMP-4-1) was obtained. Sequencing confirmed that it contained sequences from the putative translation start site to stop codon as planned. In further experiments, this clone was used as a probe in screening of genomic libraries and in Northern analyses and *in situ* hybridizations.

In the early genomic cloning experiments, a 7-kb clone containing only exons 3 to 5 was identified in a LambdaFish II Library. As the entire genomic sequence was needed for later purposes the screening experiments were expanded to another genomic library (peloBAC). A 250-kb BAC clone containing *Timp4* gene was identified and subcloned. Analysis of subclones revealed that they contained the sequence for the entire *Timp4* gene as well as putative promoter area and 3´-flanking sequence. The nucleotide sequences were uploaded to EMBL/Genbank database (accession numbers: AF345863, AF345864, AF345865 and AF345866).

Sequence analyses revealed that the murine *Timp4* gene contains 5 exons separated by relatively short introns (1.2, 0.4, 1.6 and 0.95 kb). Furthermore, exons 5 and 6 of *Syn2* gene were identified in an antiparallel orientation at 3´- and 5´-flanking regions of *Timp4* gene, respectively.

To identify the transcription initiation site of the *Timp4* gene, several parallel methods were utilized. At first, 5´-RACE turned out to be unsuccessful, as only 1–5 nucleotides could be identified upstream of the translation initiation codon in cDNA fragments obtained for total RNA from mouse heart. Next, primer extension analysis revealed two different start sites at 55 and 88 bp upstream of the ATG. Subsequently, sequence databases were analyzed to get further confirmation for the 5´-end. Two interesting EST sequences (Genbank accession numbers: AI551619, AA423441) were identified from mouse mammary gland cDNA library. Both of them shared the same 5´-end at 89 bp upstream of the ATG and contained exon 1 and partially exon 2 of the *Timp4* gene. The analysis of genomic sequence revealed a non-consensus TATA-box 14 bp and a non-consensus CAAT-box at 62 bp upstream of the putative 5´-end at the 89 bp upstream of the ATG.

After the communication *I* was submitted, another paper describing partial characterization of the murine *Timp4* gene and promoter was published by Young et al. (2002). They also reported several putative transcription initiation sites (-55, -83 and -112bp). Some of these matched closely the findings of the present study. Afterwards, the sequence information has greatly increased as projects to describe the entire mouse transcriptome have been completed (Carninci et al, 2005). The current (in January 2012) murine *Timp4* mRNA (NM_080639.3) in the reference sequence database (RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq/) of National Center for Biotechnology Information (NCBI) (Pruitt et al, 2007) indicates transcription initiation at -71bp. Taken together, the data indicate that different initiation sites are used for transcription of the murine *Timp4* gene (Figure 5.2).

Comparison of translated amino acid sequences reveals that like TIMPs 2 and 3, the murine TIMP4 shares >90% identity with the corresponding human peptide (Table 5.1). Only in TIMP1, there is a moderate difference between the murine and human sequences.

Table 5.1 Comparison of amino acid sequence homologies (identity/similarity) of mature forms of murine TIMPs and comparison between human and murine TIMPs (in bold)

	TIMP1	TIMP2	TIMP3	TIMP4
TIMP1	**70.7/84.8**	34.7/49.5	34.7/51.0	35.0/51.5
TIMP2		**98.5/99.5**	44.4/66.8	49.5/69.9
TIMP3			**97.9/98.9**	45.6/66.2
TIMP4				**90.4/94.4**

Values represent identity/similarity in percentage. Bold formatting indicates comparison between human and murine sequences, while all others are comparisons between two murine sequences. Results were generated using EMBOSS Align -tool at the website of European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align/). Peptide sequences of mature proteins were extracted from the reference sequences provided by NCBI.

All TIMPs share several structural elements: 5 coding exons, a conserved VIRAK sequence in N-terminal domain, and 12 cysteine residues forming 6 disulfide bonds. However, there are also some unique features. *TIMP1*/*Timp1* gene contains an additional non-coding exon in the 5´-region. *TIMP2*/*Timp2* and *TIMP3*/*Timp3* contain a large first intron (*TIMP2* ~51kb, *Timp2* ~32kb, *TIMP3* ~47kb, *Timp3* ~38kb). While TIMPs 2 and 4 do not have any N-glycosylation sites, TIMP1 with two and TIMP3 with one N-glycosylation site frequently exist as glycosylated. All TIMPs have a signal peptide, thus they are likely to be extracellular, although TIMP4 has also been detected intracellularly in sarcomers of cardiac myocytes (Schulze et al, 2003). While other TIMPs are soluble, TIMP3 is tightly bound to ECM molecules such as heparan sulphate and chondroitin sulphate (Yu et al, 2000).

In genomes of different species, *Timp* genes are located in an antisense orientation inside introns of *Synapsin* (*Syn*) genes. This has been shown in Drosophila (Pohar et al, 1999), and respectively in mammalian genome *Timp1* is located inside *Syn1*, *Timp3* inside *Syn3* and *Timp4* inside *Syn2* (Figure 5.1) (Karlin et al, 2002). In mammalians, only three *Synapsin* genes have been identified, and thus *Timp2* is not associated with any *Syn* gene. It seems likely that the four different *Timp* genes were generated by two duplication events during vertebrate evolution as is the case with several other gene families (Dehal & Boore, 2005; Hoffmann et al, 2011).The conservation of multiple *Timp* genes suggests that each member of the *Timp* gene family has acquired unique functions which cannot be completely compensated by the other *Timp*s. Another explanation for the conservation of several *Timp*s could be differential regulatory mechanisms. The latter is supported by variability in promoter sequences of different *Timp*s (Clark et al, 2008) and by their diverse expression patterns (Nuttall et al, 2004; Young et al, 2002). Furthermore, diverse metalloproteinase inhibitory profiles and divergent MMP independent functions support the idea that individual mammalian TIMPs have specific physiological roles and they are not just interchangeable protease inhibitors as reviewed in chapter 2.5.

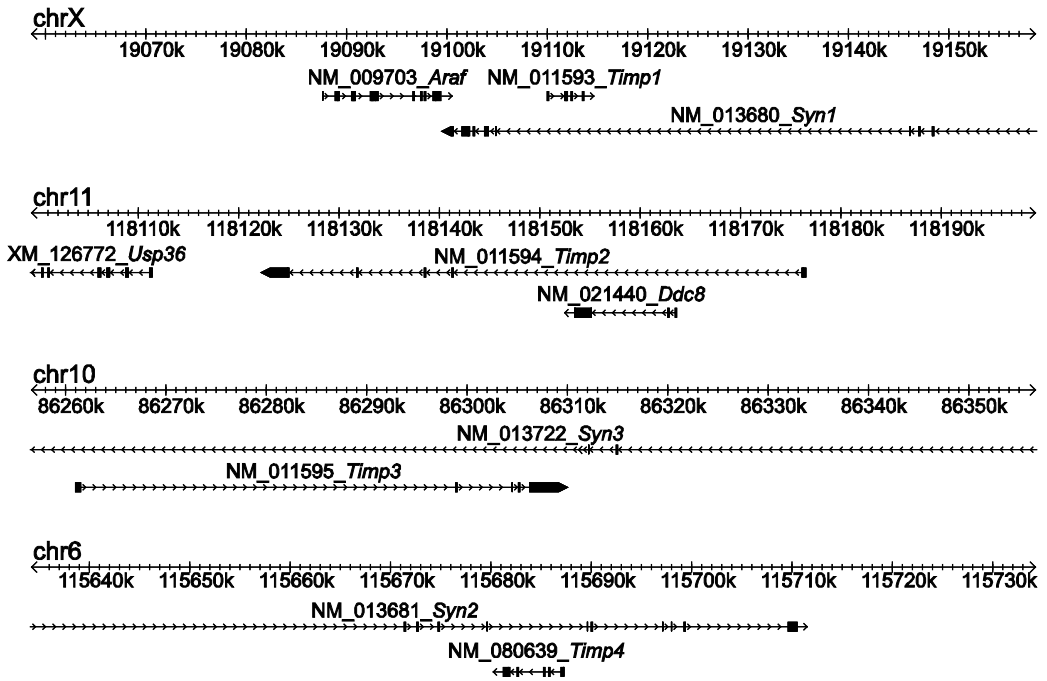

Figure 5.1 *Timp1–4* loci in respective murine chromosomes. For each *Timp* gene a 100-kb area is shown for each chromosome (chr), containing the *Timp* gene and other genes located nearby: *Araf*, v-raf murine sarcoma 3611 viral oncogene homolog; *Usp36*, ubiquitin specific peptidase 36; *Ddc8*, differential display clone 8; and *Syn*, synapsin. The codes before each gene symbol indicates Genbank accession number for corresponding mRNA sequence. Figure generated using Mouse GBrowse at Mouse Genome Informatics website (www.informatics.jax.org).

5.2 Expression pattern of *Timp4* in the mouse (*I*)

One approach towards understanding the role of *Timp4* was to study the expression pattern of *Timp4* mRNA in various tissues. For this purpose, total RNA was extracted from embryonic (E14, E16 and E18), newborn and adult murine tissues and analyzed using an RNase protection assay. The highest expression was detected in heart, ovary and brain. In heart, no expression was detected before E18, and in other embryonic tissues no expression was seen at any stage. In an earlier study (Greene et al, 1996) based on Northern analyses of human tissues, *TIMP4* expression was reported to be restricted to few tissues with low expression (kidney, pancreas, colon, and testis) and to heart with several folds higher expression. Thereafter the expression of murine *Timp4* was reported to be restricted to brain, heart, ovary and skeletal muscle (Leco et al, 1997). In contrast to the earlier reports, *Timp4* expression in the present study was also detected in lung but not in skeletal muscle. Later on, expression of *Timp4* in mouse tissues has been studied using Taqman RT-PCR method (Nuttall et al, 2004; Young et al, 2002). Obviously, the method used provided a more sensitive way for expression analysis and several new tissues were shown to express *Timp4* at moderate level (testis, kidney, liver, uterus, thymus and adrenal gland). However, these studies used mainly embryonic and newborn mouse tissues, and such findings do not necessarily correspond to the situation in adult tissues and in humans.

More recently, microarray based expression data in different human tissues and pathologies have become available. Analysis of human transcriptome provided by the GeneSapiens database (www.genesapiens.org) (Kilpinen et al, 2008), reveals the highest *TIMP4* expression in bone marrow, muscle, heart, blood vessels, adipose tissue, peripheral nervous system, respiratory system, mesothelium and adult stem cell (Figure 5.2.). Despite these findings, *Timp4* still appears to exhibit the most restricted expression pattern of the *Timp*s.

Cardiac expression of *Timp4* was further analyzed by performing *in situ* hybridizations. In E18, newborn and adult heart, diffuse distribution of *Timp4* mRNA was seen at a low level in a proportion of cardiac myocytes in atria and ventricles.

Ovarian expression of *Timp4* was analyzed in total RNAs representing six stages of the mouse oestrus cycle using Northern hybridization. The analyses revealed the highest expression level at estrus, resembling *Timp1* (Oksjoki et al, 1999), but the changes were not statistically significant. *In situ* hybridization revealed the strongest *Timp4* mRNA in mature corpora lutea, whereas lower levels were seen in the oocyte, the surrounding granulosa and theca cells of the Graafian follicle and in immature corpora lutea. This localization suggest that *Timp4* participates in ECM degradation during follicular maturation and rupture and possibly also in steroidogenesis in corpus luteum.

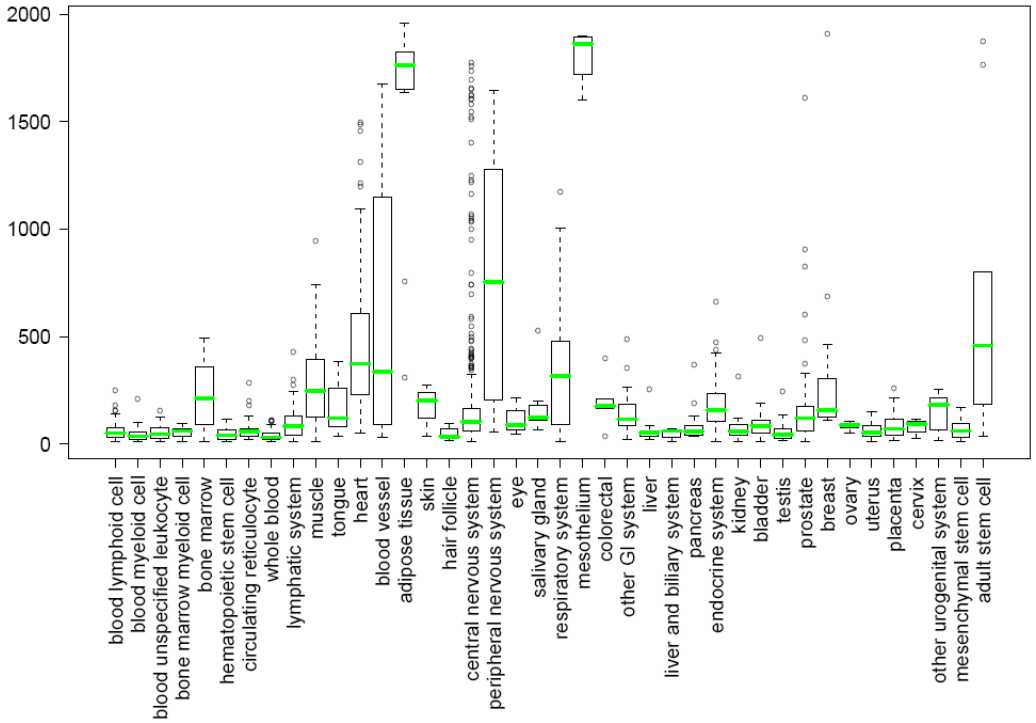

Figure 5.2. *TIMP4* expression in human tissues. Expression data from Affymetrix microarray analysis of healthy human tissues shown as a standard box-whisker plot. Figure generated using the GeneSapiens database at www.genesapiens.org

5.3 Regulatory elements in the promoter of *Timp4* gene (*I*)

The regulation of *Timp4* expression is poorly understood. In the present study, primer extension analyses revealed two different transcription start sites (55 and 89 bp upstream of ATG) of the murine *Timp4* (Figure 5.3.). Computational analysis revealed a non-consensus TATA-box 14 bp and a non-consencus CAAT-box at 62 bp upstream of the predicted transcription initiation (at 89 bp upstream of ATG). Analysis of the promoter region of murine *Timp4* gene by Young et al.(2002) revealed relatively few identifiable transcription factor-binding consensus motifs (motifs for GATA-, myogenin-, Ets-, Sp1- and CCAAT-binding proteins). In promoter construct experiments, mutation of the CCAAT box doubled and mutations of initiator-like element or Sp1 motif significantly decreased expression in fibroblastic cell line, but no other motifs were tested (Young et al, 2002). Based on the expression pattern of endometrial TIMP4, Pilka et al. (2004) suggested that estrogen could regulate *TIMP4* mRNA expression. Accordingly, they identified a sequence with homology to the consensus sequence of estrogen receptor element in promoter region of *TIMP4* gene.

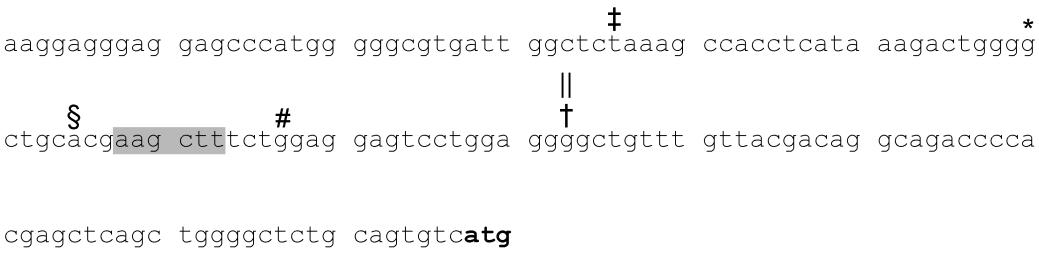

Figure 5.3. Predicted transcription start sites of the murine *Timp4* gene. Translation initiation codon (atg) bolded. * and † indicate transcription initiation sites as described in communication *I*; and ‡, § and || as described by Young *et al*. (2002). # indicate start site of the *Timp4* mRNA reference sequence (NM_080639.3) at NCBI. HindIII restriction site used for cloning of the *Timp4* knockout construct is shaded.

Regulation of TIMP4 expression has also been studied using other approaches. The effects of different cytokines on TIMP4 expression have been analyzed in cell culture systems. When stimulated with TNF and interleukin-1β, neonatal rat ventricular myocytes transiently increased TIMP4 protein expression, while mRNA expression remained unchanged (Li et al, 1999). Interestingly, this discrepancy of mRNA and protein expression has been reported also in cardiac tissue samples of patients with aortic stenosis suggesting an important role for posttranscriptional regulation (Fielitz et al, 2004). Another aspect of posttranscriptional regulation was detected in human breast carcinoma: while *TIMP4* mRNA was localized only to stromal fibroblasts surrounding the carcinoma, the protein was mainly present in the malignant epithelial cells (Jiang et al, 2001). Analogously, in human endometrial tissue *TIMP4* mRNA was found in stromal cells, whereas TIMP4 protein was detected in epithelial cells (Pilka et al, 2006). These observations suggest that the regulation of TIMP4 activity in tissues involves not only regulation at transcriptional level, but also posttranscriptional modulation and intercellular transportation from stromal to epithelial cells.

5.4 Generation of *Timp4* knockout mice (*II*)

Once the cloning of *Timp4* gene was completed, a targeting vector was designed for production of *Timp4* deficient mice. Two relatively long arms (2.6 and 2.2kb) of flanking DNA as well as neomycin resistance and thymidine kinase selection cassettes were used to construct a plasmid, which was electroporated into mouse ES cells after linearization and purification. Using Southern blotting, only one clone (of the total number of 216 isolated clones) proved out to be positive for the correct targeting event. This clone was chosen for further culture and subsequent blastocyst injections, which resulted in four high-percentage chimeric male mice, two of which frequently produced heterozygous pups.

Breeding of heterozygotes resulted in pups homozygous (*Timp4*$^{-/-}$), heterozygous (*Timp4*$^{+/-}$) and negative (*Timp4*$^{+/+}$) for the *Timp4* targeting mutation. The predicted

mutant allele lacks exons 1 to 3 of the *Timp4* gene. The upstream end of the deleted region is located at the 3´-end of a HindIII restriction site (-74bp, Figure 5.3.). After gene-targeting, most of the predicted transcription initiation sites are deleted and also the others are possibly disrupted. Thus, *Timp4*$^{-/-}$ mice were not supposed to produce any *Timp4* RNA. Indeed, using *Timp4* cDNA as a probe, no signal was detected when Northern blotting was performed on RNAs extracted from *Timp4*$^{-/-}$ hearts. In hearts of heterozygous mice, *Timp4* expression was decreased to half of the normal.

5.4.1 Lack of *Timp4* has no obvious effects on mouse development, growth or fertility (*II* and *III*)

Breeding of heterozygous mice resulted in pups exhibiting different genotypes (*Timp4*$^{+/+}$, *Timp4*$^{+/-}$ and *Timp4*$^{-/-}$) in a Mendelian, 1:2:1, ratio with 1:1 sex distribution. No pathologies were observed during development of *Timp4* deficient mice. Also survival rates and body weights were comparable between the genotypes.

Special attention was paid to the evaluation of fertility, because relatively high expression levels of *Timp4* in the ovary and testis suggested that *Timp4* could affect reproductive functions. At 5 months, *Timp4* deficiency did not affect ovarian histology or estrus cycle suggesting that lack of TIMP4 does not affect steroid production of corpus luteum. Breeding of homozygous (*Timp4*$^{-/-}$ x *Timp4*$^{-/-}$), heterozygous (*Timp4*$^{+/-}$ x *Timp4*$^{+/-}$) and control (*Timp4*$^{+/+}$ x *Timp4*$^{+/+}$) breeding pairs gave rise to litters of same size strongly supporting normal fertility of *Timp4* deficient mice. Finally, Mendelian ratio of pups in breedings of heterozygous mice implies that *Timp4* does not affect the implantation process of embryos or their further survival during gestation.

5.4.2 Characterization of the cardiac structure and function in *Timp4*$^{-/-}$ mice (*II*)

When producing *Timp4*$^{-/-}$ mice, a cardiac phenotype was expected as the previous literature had shown high expression levels of *Timp4* in the heart. Furthermore altered expression levels of *Timp4* had been reported in different types of heart diseases. Therefore, particular attention was paid to the heart and the entire cardiovascular system when searching for a phenotype for *Timp4* knockout mice.

The development and growth of *Timp4*$^{-/-}$ and *Timp4*$^{+/+}$ mice was followed up to 16 months. During this follow-up the body weights and heart-to-body weight ratios remained comparable between genotypes. When measured at the age of 5 months, blood pressure and heart rate were similar in knockout and control mice. Cardiac histology was carefully evaluated at the age of 5 and 16 months. No differences were seen in the size of cardiac myocytes or in the amount of fibrosis. By measuring cardiac hydroxyproline content, the amount of collagen was shown to be comparable in *Timp4*$^{-/-}$ and *Timp4*$^{+/+}$ hearts.

As no differences were seen in *Timp4*$^{-/-}$ hearts by 16 months, possible compensation by other *Timp*s was tested next. However, in Northern blot or Taqman RT-PCR analysis of total heart RNA extracted from 2 and 5 months old mice, no differences were seen in cardiac expression of *Timp*s 1, 2 or 3 between the genotypes.

Since it became obvious that *Timp4* deficiency does not affect murine hearts during development or normal adulthood, the next stage was to study *Timp4*$^{-/-}$ hearts in situations with different types of cardiac stress. The first step was to investigate ageing mice. For this, 20 month old *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice were subjected for measurements of cardiac function by *in vivo* echocardiography. In *Timp4*$^{-/-}$ mice a poor myocardial performance index was detected, suggesting reduced cardiac function. Nevertheless, other functional parameters, fractional shortening and E/A –ratio, remained comparable between the genotypes. On the other hand, coronary flow reserve, a measurement of coronary microvascular function, was decreased in *Timp4* knockout mice. Echocardiography also demonstrated increased septal and posterior wall thicknesses as well as increased left ventricular mass in *Timp4*$^{-/-}$ hearts. Using histomorphometry myocyte cross-sectional area was measured and shown to be similar in *Timp4*$^{-/-}$ and control mice. Although fibrosis was increased in 20-month old hearts, the levels were comparable between aged *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice.

5.4.3 Cardiac response of *Timp4* knockout mice to pressure overload (*II*)

As aging *Timp4*$^{-/-}$ hearts showed deteriorated cardiac function, *Timp4* deficient mice were subjected to an aortic banding experiment, which results in cardiac pressure overload. In control mice, aortic banding launches processes leading to extensive ventricular hypertrophy in order to compensate for the pressure overload. However, because this pathologic overload is permanent, the result of compensatory mechanisms is an excessive hypertrophy which leads to heart failure. Eventually the left ventricle dilates and ventricular wall becomes thinned and fibrotic. Significant mortality is evident by 16 weeks after aortic banding. By performing aortic banding experiment for *Timp4*$^{-/-}$ and control mice, a better understanding of the role of TIMP4 in such processes as cardiomyocyte hypertrophy and myocardial fibrosis was expected.

Aortic banding was performed for *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice at the age of 8 weeks. Survival rate of *Timp4* deficient mice did not differ from that of control mice during the 12 week follow-up. Furthermore, no significant differences were seen in cardiac function as measured by *in vivo* echocardiography at 1, 3, 6 and 12 weeks after aortic banding. At 6 weeks, histological evaluation of cardiac hypertrophy and fibrosis did not reveal differences between the genotypes. Correspondingly, the expression levels of natriuretic peptides, ANP and BNP, as well as major cardiac collagens, types I and III, were comparable in *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ hearts.

As no phenotype was identified in the aortic banding experiment, it was hypothesized that lack of TIMP4 could be compensated by *Timp* genes. To examine this possibility, cardiac

expression levels of the other *Timp*s were assessed by Taqman RT-PCR method. While the expression levels of *Timp1*, *Timp2* and *Timp3* were identical in control hearts (sham-operated), *Timp2* expression was significantly increased after aortic banding ($P<0.05$). This suggests that after aortic banding *Timp2* is able to compensate for the lack of *Timp4*. Among TIMPs, the structure of TIMP2 is the most similar to TIMP4, and the present study indicates that it is also capable of taking up the physiologic role of TIMP4 in heart

5.4.4 *Timp4* deficient mice are predisposed to fatal cardiac rupture after myocardial infarction that can be rescued by MMP inhibition (*II*)

As aging revealed only a moderate dysfunction in *Timp4* null mice and response to pressure overload was not altered, *Timp4* deficiency was next studied in another type of cardiac challenge, i.e. myocardial infarction. In earlier reports, *Timp4* downregulation has been found in myocardial infarction models of different mammalians (Mukherjee et al, 2003; Wilson et al, 2003) and in patients with ischemic cardiomyopathy (Li et al, 1998). In response to ischemia-reperfusion injury, *Timp4* is immediately released from the myocardium (Schulze et al, 2003). In the present study, *Timp4* null mice were subjected to experimental myocardial infarction by LAD ligation.

Ligation resulted in a large myocardial infarction and progressive left ventricular dilation, which were comparable in $Timp4^{+/+}$ and $Timp4^{-/-}$ mice. At 3 to 7 days after infarction, survival of *Timp4* deficient mice was significantly deteriorated compared to $Timp4^{+/+}$ mice. At autopsy, a ventricular rupture was identified as a cause of death in a majority of cases. At 7 days after infarction, a severe cardiac dysfunction was evident by echocardiography and *in vivo* hemodynamics, but no differences were found between the genotypes.

Next, a broad spectrum MMP-specific inhibitor (PD166793; MMPi) was administered to $Timp4^{+/+}$ and $Timp4^{-/-}$ mice, which were subjected to LAD ligation. The increased mortality in *Timp4* knockout mice was completely rescued by MMPi. Furthermore, $Timp4^{-/-}$ / $Mmp2^{-/-}$ double knockout mice were generated and subjected to LAD ligation. Similar to MMPi, the genetic ablation of *Mmp2* also rescued the icreased mortality in *Timp4* deficient mice. Thus, TIMP4 likely functions as an essential control of MMPs in the healing process after myocardial infarction. This finding is in line with earlier observations in mice on the importance of specific MMPs in cardiac rupture (Hayashidani et al, 2003; Matsumura et al, 2005; Tao et al, 2004).

5.4.5 Wound healing in *Timp4* knockout mouse (*III*)

No earlier studies are available on the role of TIMP4 in wound healing. In chronic dermal ulcers, however, TIMP4 has been detected in stromal cells adjacent to endothelial cells (Vaalamo et al, 1999). Considering the importance of blood coagulation and inflammation in wound healing, TIMP4 could play an important role, as it is the major TIMP in platelets (Radomski et al, 2002) and it is expressed by some inflammatory cells (*IV*). Wound healing was chosen as the first experimental challenge for *Timp4* null mice,

as physiologic healing utilizes several biological processes which are important also in various pathologies.

To study the effects of *Timp4* deficiency in cutaneous wound repair, healing of excisional dermal wounds was monitored in 7-month old *Timp4* deficient and control mice. Re-epithelialization was significantly retarded in *Timp4*$^{-/-}$ wounds at 7 days. However, complete re-epithelialization was achieved in both genotypes at 9 days after wounding. Using immunohistological stainings the number of capillaries and neutrophils in healing tissue were studied, but no differences were detected. Thus, lack of TIMP4 is likely to alter MMP/TIMP balance necessary for optimal migration of epidermal keratinocytes during dermal wound healing.

5.4.6 *In vivo* angiogenesis is not altered in *Timp4*$^{-/-}$ mice (*III*)

To better understand these findings, the effect of *Timp4* deficiency on angiogenesis was examined *in vivo* using Gelfoam sponges implanted subcutaneously into *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. FGF2 was used as an angiogenic stimulus. At 14 days post-implantation, the amount of new vessels in both genotypes was identical as determined by histomorphometric analysis of endothelial CD31 immunostaining. Other studies have been published on the effects of exogenous TIMP4 on angiogenesis. In *in vitro* models, recombinant TIMP4 has been shown to inhibit endothelial cell invasion and tubulogenesis (Bayless & Davis, 2003; Lafleur et al, 2002). However, in another study, exogenous TIMP4 did not affect endothelial cell proliferation *in vitro* and did not inhibit embryonic angiogenesis *in vivo* (Fernández & Moses, 2006).

5.4.7 Melanoma tumor growth in the absence of *Timp4* (*III*)

Several studies have been published on the role of TIMP4 in cancer. In clinical studies, either upregulation or downregulation of *TIMP4* has been implied in different types of tumors. In *in vitro* -models, anti-tumor effects such as inhibition of invasion (Wang et al, 1997) or apoptosis of transformed cells (Tummalapalli et al, 2001), have been observed in response to higher levels of TIMP4. In nude mice, overexpression of TIMP4 in human breast cancer tumors decreased tumor growth, while increased TIMP4 in circulation either decreased or increased tumor growth depending on tumor type (Celiker et al, 2001; Jiang et al, 2001). To clarify the role of TIMP4 in cancer, growth of melanoma tumors was studied in *Timp4* deficient and control mice.

B16F10 melanoma cells were subcutaneously implanted into *Timp4* knockout and control mice. Mice were followed until 17 days post-implantation, but no differences were observed in tumor incidence, latency or growth rate between *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. Because expression of *Timp4* has not been tested in the subcutaneous space, the experiment was repeated at another site known to express *Timp4*. Thus, tumor cells were next injected into skeletal muscle. B16F1 cells were used in this case, as earlier studies had demonstrated their growth at this site. Nevertheless, no

differences were seen in the growth of intramuscular tumors between *Timp4* null mice and controls. Furthermore, angiogenesis was evaluated by factor VIII related antigen immunostainings. No significant differences were seen in the number of capillaries in *Timp4* knockout and control mice. This supports the previous finding of unaltered angiogenesis in the Gelfoam model.

5.4.8 *Timp4* deficiency increases pulmonary metastases (*III*)

Although *Timp4* deficiency of the host did not affect melanoma growth when implanted subcutaneously or intramuscularly, *Timp4* deficiency in cancer biology needed to be investigated in more depth. An experimental metastasis model was chosen for the next investigation, as biology of this model differs in many ways from the tumor growth experiments described above. In this model, lung colonization is determined by the efficiencies of trapping of melanoma cells in mouse lungs as well as extravasation and initial survival of solitary cells (Cameron et al, 2000). Moreover, each colony originates from a single cell. In tumor growth experiments, a relatively high number of cells, i.e. $\sim 10^5$ cells, was used which might not be the most sensitive assay.

Experimental metastasis was performed by injecting B16F10 melanoma cells into tail vein of *Timp4*$^{+/+}$ and *Timp4*$^{-/-}$ mice. When pulmonary metastases were examined 14 days post-injection, *Timp4*$^{-/-}$ lungs showed a two-fold increase in the number of melanotic surface colonies. Histomorphometric analysis of lungs revealed a comparable increase in the ratio of tumor tissue to total lung tissue. For differences in angiogenesis or proliferation, factor VIII related antigen and Ki67 immunostainings were performed, respectively, but no significant differences were seen between *Timp4* null and control lungs.

Because the read-out in our metastasis experiment was the end-point at 14 or 21 days post-injection, it did not reveal any information about the early events of metastasis, which *Timp4* deficiency affects. To get more insight, a lung trapping assay was performed in order to differentiate the initial steps of metastasis. Although no significant differences were seen, some trends were obvious. Initial trapping was not altered, as the plating efficiency at 15 min time point was nearly identical between the genotypes. After the 48 h time point, a trend of increased plating efficiency in knockout mice was observed. Thus, the increased metastasis in *Timp4* deficient lungs is likely determined between 15 min and 48 h time points, a period when single tumor cells either undergo apoptosis or survive and will later start proliferating and forming tumor colonies. The 8 d time-point showed more than a four-time increase of metastatic colonies in *Timp4*$^{-/-}$ lungs. However, at this stage most of the tumor colonies in lungs were composed of several cells, which will result in multiple colonies on dish. Thus the 8 d result was not comparable with earlier time-points as it did not represent the number of tumor colonies in lungs in same manner as earlier time-points.

5.5 TIMP4 is associated with inflammation in human cardiovascular pathology (*IV*)

Although the present study and several other reports have indicated a role for TIMP4 in cardiovascular diseases, little information was available specifically about TIMP4 in human vascular diseases. Originally, Dollery et al. (1999) showed that TIMP4 is overexpressed throughout vascular wall after balloon injury of carotid artery in rats. This kind of injury leads not only to ECM remodelling of vascular wall but also to local inflammatory reaction. TIMP4 has been associated with the latter in other circumstances like myocardial reperfusion injury (Schulze et al, 2003) and in adjuvant-induced arthritis (Celiker et al, 2002) in rats. When expanding the current studies to human vascular diseases, giant cell arteritis and atherosclerosis were chosen as inflammation plays a crucial role in the pathogenesis of these diseases.

A valuable set of atherosclerotic coronary artery samples was collected from patients at the time of heart transplantation. Donor hearts, inappropriate for transplantation because of the size or tissue-type mismatch, served as control samples. At histological level, the control arteries were free of atherosclerosis and did not show any immunostaining for inflammatory cells. In immunohistochemical staining for TIMP4, positive cells were detected in the medial smooth muscle cell layer and in capillaries in the adventitia, i.e. the outermost layer of the vascular wall. In coronary arteries with intermediate or advanced type of atherosclerotic lesions, strong staining for TIMP4 was detected also in the intima in the same areas where macrophages and CD3+ T cells were located.

Once TIMP4 was shown to be involved in inflammation in coronary arteries, it was tested whether this finding represents a more widespread phenomenon. TIMP4 localization was investigated in histologically healthy temporal arteries and in arteries exhibiting giant cell arteritis. In contrast to coronary arteries, healthy temporal arteries did not show any staining for TIMP4. However, in inflamed arteries, TIMP4 staining was detected in intimal areas where macrophages were dominating.

Next, the association of TIMP4 and inflammation was tested in other parts of the cardiovascular system than in the artery wall. In endomyocardial biopsy samples from human heart allografts exhibiting signs of rejection, TIMP4 staining was seen in infiltrating leucocytes. At the same time, TIMP4 was also localized to cardiac myocytes in areas with and without leucocyte infiltration.

The function of TIMP4 in the context of vascular inflammation is unknown. Towards getting more information, serial sections of coronary arteries were analyzed for TIMP4, TIMP3 and caspase 3. As TIMP3 has been linked to apoptosis in other systems, it was hypothesized that it is proapoptotic also in atherosclerotic lesions. Indeed, TIMP3 and caspase 3 were detected in and around the necrotic lipid core, while TIMP4 was only present in areas surrounding the necrotic core. Thus, TIMP4 and TIMP3 are differentially located in atherosclerotic lesions and may possess different roles in inflammation-induced apoptosis during coronary atherogenesis.

5.5.1 TIMP4 is synthesized by human inflammatory cells *in vitro* (*IV*)

To further confirm coupling of TIMP4 and inflammation, experiments were performed to test TIMP4 production by human inflammatory cells *in vitro*. Using Western blotting and immunocytochemistry, TIMP4 was shown to be produced by cultured human monocytes, macrophages and mast cells as well as isolated lymphocytes.

Three inflammatory cell types, macrophages, T lymphocytes and mast cells, contributing to the pathogenesis of atherosclerosis were shown to be able to produce TIMP4. This supports the previous finding about co-localization of TIMP4 and inflammatory cells in clinical specimens representing various cardiovascular diseases with an inflammatory component. Together these findings suggest that in these diseases, inflammatory cells in vascular wall and myocardium produce TIMP4 protein. As TIMP4 is a soluble protein, levels of circulating TIMP4 may signal a state of vascular inflammation. Next, this possibility should be tested in diseases such as vasculitis and atherosclerosis.

5.6 General discussion

5.6.1 Molecular biology and genetics of TIMP4

In mice and humans the TIMP gene family comprises four non-identical members in contrast to one member in *Drosophila*. This supports the theory that evolution of the mammalian genome has involved two duplication events (Dehal & Boore, 2005; Hoffmann et al, 2011). These duplication events have increased the copy number of many genes four-fold and made it possible for individual genes to develop into different directions with different regulatory regions, functions and expression patterns. Yet, this has allowed for the gene products to maintain partially overlapping functions as is clearly demonstrated for the four members of the TIMP gene and protein family; e.g. redundancy between TIMPs is demonstrated by compensated upregulation of *Timp2* in *Timp4* deficient mice after aortic banding. Analogously, redundancy or compensation by other TIMPs is likely also in *Timp1* and *Timp2* knockout mouse models, which show obvious phenotypes only when they are challenged. However, conservation of all four mammalian TIMPs in an active form verifies that each of them has also gained some unique features such as different inhibitory profiles especially for MT-MMPs and ADAMs as well as differently regulated expression in various physiologic and pathologic situations.

The mouse and human *TIMP1*, *3* and *4* genes reside in an antisense orientation within the fifth intron of the three *Synapsin* genes found in mammals (Figure 5.1). It is interesting to note a similar locus organization also in *Drosophila*; the single *TIMP* gene is located with the single *Synapsin* gene (Pohar et al, 1999). Yet, no information is available on possible functional relationships of the *TIMP* and *Synapsin* genes. Now that the important role of gene methylation and transcriptional activity of genomic regions is becoming available,

it will be possible to study any relationships that may exist between the expression levels of specific TIMPs and synapsins. For example, it is quite possible that active transcription of the *Synapsin* gene resulting in opening of the genomic structure of the locus also opens the *TIMP* gene within intron 5 for transcription factor binding and subsequent transcription. Databases, such as GeneSapiens (see Fig. 5.2) will provide a novel approach to investigate whether such correlations really exist. The differential expression patterns of the four *TIMP* genes suggest they all are controlled by specific mechanisms, but the contribution of *Synapsin* gene regulation to this control has not been studied.

Interestingly the genomic locus of *Timp2* differs from that of other *Timp*s, as in the mouse or human genomes there are no *Synapsin* genes in the same chromosome as *Timp2*. Moreover, *Timp2* contains another gene, *Ddc8*, within its first intron in the same orientation. Furthermore, Jaworski et al. (2007) have reported that in the mouse brain the expression pattern of *Ddc8* mimics that of *Timp2*. Interestingly, a portion of *Timp2* mRNAs in the brain contains also *Ddc8* sequence likely through alternative splicing.

As regulatory elements of genes reside not only in the promoter regions but within genes (and sometimes considerable distances outside the gene region) one should also revisit the *Timp* knockout mice and analyze whether the expression levels of the *Synapsin* genes spanning the mutated *Timp* genes remain unaltered. In principle modification of *Timp* gene structure by the targeting event could also disturb the expression of the *Synapsin* gene.

5.6.2 Role of TIMP4 in matrix turnover and tissue remodelling

The importance of matrix turnover in normal tissues and under specific physiologic and pathologic situations has been well documented. The importance of the ECM to the integrity of essentially all tissues requires a tightly regulated process whereby different matrix degrading enzymes and their inhibitors balance degradation of different matrix components with synthesis of new matrix in specific sites at specific times and at different rates. As discussed earlier, degradation of collagenous matrix structures (fibers, fibrils, networks) presents a special challenge and requires specific sets of proteinases, MMPs and ADAMs, and their inhibitors, TIMPs. Thus TIMPs are potentially key regulators of ECM turnover both under several physiological and pathological conditions. However, it has been difficult to assess the role of individual TIMPs under these different conditions in different tissue locations.

The approach selected in the present study, production of genetically modified mice with inactivated *Timp4* genes, offered a new way to systematically investigate the role of an individual TIMP in mice. Based on the more restricted tissue distribution of *Timp4* compared to other *Timp*s one might have expected *Timp4* knockout mice to develop a pathological phenotype. Against this assumption, however, *Timp4* knockout mice developed, grew and reproduced normally. This clearly indicates that the activities of

other *Timp* family members are capable of compensating for the lack of *Timp4* activity. The existence of such a back-up system serves as another proof for the importance of *Timp*s as inhibitors of MMPs and ADAMs; despite the divergence of the four *Timp*s they retain sufficient redundancy to maintain their key biological function.

Based on the relative abundance of *Timp4* expression in the heart and vascular system, the challenging experiments of the present study focused on these tissues and on processes where tissue remodeling is increased (wound healing and tumor metastasis). The two latter processes serve as examples of rapid degradation of ECM components where TIMPs have potentially an important role to regulate matrix degradation and synthesis.

The results of the present study indeed demonstrate that TIMP4 has a special role in regulation of tissue repair processes in the heart, but also in healing wounds and metastases in the mouse. Further studies are needed to determine the exact role of TIMP4 in human disease pathologies. As a first step towards this goal, the involvement of TIMP4 in inflammatory processes of vascular wall was studied in selected human samples. The observed upregulation of TIMP4 in cardiovascular disorders involving inflammation suggests it could be of use as a novel systemic marker for different types of vasculites. However, future studies of TIMP4 involvement in human vasculitis and atherosclerosis are needed to verify these observations. It is particularly important to study whether TIMP4 is also involved in the pathogenesis of atherosclerosis and in healing of myocardial infarction. The same is true for the potential involvement of TIMP4 in cancer biology, especially metastatic spreading of tumors. If further studies confirm the beneficial effects of TIMP4 preventing cardiac rupture after myocardial infarction and inhibiting tumor metastasis, overexpression of TIMP4 could provide novel therapeutic avenues towards treatment of these conditions. Obviously, further experimentation is needed to determine how an increase or normalization of TIMP4 levels in these disease processes can be achieved and what are the long term consequences of such treatments.

6. SUMMARY AND CONCLUSIONS

Based on the results of the present investigation, the following conclusions can be drawn:

1. The structure of murine *Timp4* cDNA genomic locus resembles that in other species and of the other *Timp*s. In mice the highest *Timp4* expression was detected in heart, ovary and brain.

2. The generation and analysis of *Timp4* knockout mice revealed that *Timp4* deficiency has not obvious effect on development, growth or fertility.

3. In experimental cardiac pressure overload model, *Timp4* deficiency is compensated by *Timp2* overexpression. In contrast, after experimental myocardial infarction, *Timp4* deficiency results in increased mortality due to increased susceptibility for cardiac rupture.

4. *Timp4* deficiency results in early retardation of re-epithelization of cutaneous wounds but the time to reach complete re-epithelization remains unaltered. Melanoma tumor growth was similar in *Timp4* deficient and control mice. Despite this, lung metastasis of melanoma cells was significantly increased in *Timp4* null mice.

5. In human patient material, TIMP4 was shown to be present in different cardiovascular pathologies with inflammatory component, i.e. giant cell arteritis, atherosclerotic coronary arteries and heart allografts exhibiting signs of chronic rejection.

The results of the present study thus suggest that TIMP4 has a special role in regulation of tissue repair processes in the heart, but also in healing wounds and metastases. Further studies are needed to determine whether analysis and modulation of the TIMP4 levels in human disease pathologies could be used for diagnostic and therapeutic purposes.

7. ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Genetics, University of Turku. In addition, important parts of this study were performed at the Ontario Cancer Institute, Toronto. Professors Jukka Finne, Eero Vuorio, Risto Penttinen, Klaus Elenius and Rama Khokha are acknowledged for providing the excellent facilities and encouraging atmosphere for research.

My deepest gratitude belongs to my supervisor Professor Eero Vuorio for sharing his expertise in science and guiding me through different steps of this project. As well, I am grateful to my other supervisor Docent Hannu Järveläinen for his support and enthusiasm during the later parts of this study. Their never-ending optimism has been essential to complete this project. I also thank Eero for revising the language of this thesis.

Professors Yrjö T. Konttinen and Seppo T. Nikkari, the official reviewers of this thesis, are acknowledged for their constructive criticism.

I want to thank the Turku Doctoral Programme of Biomedical Sciences (TuBS) and its director Professor Olli Lassila for all the support during these years.

I have been privileged to have enthusiastic scientists in the supervisory committee of this thesis. Professor Veli-Matti Kähäri's expertise in the TIMP field has been of great help during this project and when finishing this thesis. Professor Matti Poutanen, a former member of the supervisory committee, is acknowledged for his advice in designing gene targeting experiments. I want to thank Docent Eero Jokinen for valuable comments of a clinician during this project. I owe my special thanks to Dr Otto Rahkonen, who originally introduced me into the molecular biology of cardiovascular system and especially into the fascinating projects to characterize TIMP4.

During the years 2004–2006, I had an opportunity to join Professor Rama Khokha's research group at the Ontario Cancer Institute. I will always be grateful for this experience that taught me a lot of science and life. Important parts of this thesis had never been performed without this collaboration.

I wish to thank Drs Otto Rahkonen, Riku Kiviranta, Jukka Morko, Sanna Oksjoki, Kirsi Joronen, Anna-Marja Säämänen, Heli Salminen-Mankonen, Mirva Söderström and Hannele Uusitalo-Järvinen who once formed the Gruppo Vuorio. You are all acknowledged for the enjoyable moments in the lab. I also thank Tuula Oivanen and Merja Lakkisto for expert technical help, Rakel Mattsson for secretarial assistance and all the senior and junior colleagues at the Department of Medical Biochemistry and Genetics. I am also grateful for sharing pleasant time inside and out the lab in Toronto with Dr Zamaneh Kassiri, Dr David Smookler, Marco Di Grappa, Dr William Cruz-Muñoz and all the other members of the Khokha Lab. I owe my thanks to the personnel of the animal facilities in Turku and in Toronto and for the Umeå Transgene Core Facility and the Turku Trasgene Core facility.

Some co-authors have not been mentioned earlier, but I owe many thanks to you all: Docent Ville Kytö, Docent Antti Saraste, Dr Mervi Toriseva, Sari Pakkanen, Annele Sainio, Dr Michael Husheem, Docent Jukka Laine, Docent Harri Hakovirta, Dr Mikko Mäyränpää, Professor Petri Kovanen, Dr Trevor McKee, Dr Gavin Oudit and Professor Peter Liu.

All my friends outside the lab life are sincerely thanked for being there and sharing some special moments. Specially, I want to thank Tuomas Lehtinen, Jarkko Heino and Kari and Johanna Syvänen for memorable moments in medical school and thereafter; Seppo Fagerlund for friendship and for unforgettable times outdoors; and Sakari Kettunen for long-lasting friendship and outstanding excursions to mountains and elsewhere.

I am grateful to my parents, Anja and Hannu, my sisters, Soili and Outi and their families, and my brother Jaakko for their support and patience during these years and before. Finally, I want to thank Anniina for all the support and love and for sharing life with me and our amazing daughter.

Turku, August 2012

Ilpo Koskivirta

8. REFERENCES

Ahokas K, Lohi J, Lohi H, Elomaa O, Karjalainen-Lindsberg ML, Kere J, Saarialho-Kere U (2002) Matrix metalloproteinase-21, the human orthologue for XMMP, is expressed during fetal development and in cancer. *Gene* **301:** 31-41

Ahonen M, Poukkula M, Baker AH, Kashiwagi M, Nagase H, Eriksson JE, Kähäri VM (2003) Tissue inhibitor of metalloproteinases-3 induces apoptosis in melanoma cells by stabilization of death receptors. *Oncogene* **22:** 2121-2134

Aimes RT, Quigley JP (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* **270:** 5872-5876

Amour A, Knight C, Webster A, Slocombe P, Stephens P, Knäuper V, Docherty A, Murphy G (2000) The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. *FEBS Lett* **473:** 275-279

Amour A, Knight CG, English WR, Webster A, Slocombe PM, Knäuper V, Docherty AJ, Becherer JD, Blobel CP, Murphy G (2002) The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. *FEBS Lett* **524:** 154-158

Amour A, Slocombe P, Webster A, Butler M, Knight C, Smith B, Stephens P, Shelley C, Hutton M, Knäuper V, Docherty A, Murphy G (1998) TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* **435:** 39-44

Apte SS (2009) A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. *J Biol Chem* **284:** 31493-31497

Aumailley M, Gayraud B (1998) Structure and biological activity of the extracellular matrix. *J Mol Med (Berl)* **76:** 253-265

Balbín M, Fueyo A, Knäuper V, López JM, Alvarez J, Sánchez LM, Quesada V, Bordallo J, Murphy G, López-Otín C (2001) Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. *J Biol Chem* **276:** 10253-10262

Balbín M, Fueyo A, Tester AM, Pendás AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, López-Otín C (2003) Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* **35:** 252-257

Bateman JF, Boot-Handford RP, Lamandé SR (2009) Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations. *Nat Rev Genet* **10:** 173-183

Bayless KJ, Davis GE (2003) Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. *Biochem Biophys Res Commun* **312:** 903-913

Beighton P, De Paepe A, Steinmann B, Tsipouras P, Wenstrup RJ (1998) Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). *Am J Med Genet* **77:** 31-37

Bigg HF, Morrison CJ, Butler GS, Bogoyevitch MA, Wang Z, Soloway PD, Overall CM (2001) Tissue inhibitor of metalloproteinases-4 inhibits but does not support the activation of gelatinase A via efficient inhibition of membrane type 1-matrix metalloproteinase. *Cancer Res* **61:** 3610-3618

Bigg HF, Shi YE, Liu YE, Steffensen B, Overall CM (1997) Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A. TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. *J Biol Chem* **272:** 15496-15500

Bode W, Gomis-Rüth FX, Stöckler W (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett* **331:** 134-140

Brauer R, Beck IM, Roderfeld M, Roeb E, Sedlacek R (2011) Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen. *BMC Biochem* **12:** 38

Brew K, Nagase H (2010) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim Biophys Acta* **1803:** 55-71

Byers PH, Krakow D, Nunes ME, Pepin M, genetics Acom (2006) Genetic evaluation of suspected osteogenesis imperfecta (OI). *Genet Med* **8:** 383-388

Cameron MD, Schmidt EE, Kerkvliet N, Nadkarni KV, Morris VL, Groom AC, Chambers AF, MacDonald

IC (2000) Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. *Cancer Res* **60:** 2541-2546

Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, Kodzius R, Shimokawa K, Bajic VB, Brenner SE, Batalov S, Forrest AR, Zavolan M, Davis MJ, Wilming LG, Aidinis V, Allen JE, Ambesi-Impiombato A, Apweiler R, Aturaliya RN, Bailey TL, Bansal M, Baxter L, Beisel KW, Bersano T, Bono H, Chalk AM, Chiu KP, Choudhary V, Christoffels A, Clutterbuck DR, Crowe ML, Dalla E, Dalrymple BP, de Bono B, Della Gatta G, di Bernardo D, Down T, Engstrom P, Fagiolini M, Faulkner G, Fletcher CF, Fukushima T, Furuno M, Futaki S, Gariboldi M, Georgii-Hemming P, Gingeras TR, Gojobori T, Green RE, Gustincich S, Harbers M, Hayashi Y, Hensch TK, Hirokawa N, Hill D, Huminiecki L, Iacono M, Ikeo K, Iwama A, Ishikawa T, Jakt M, Kanapin A, Katoh M, Kawasawa Y, Kelso J, Kitamura H, Kitano H, Kollias G, Krishnan SP, Kruger A, Kummerfeld SK, Kurochkin IV, Lareau LF, Lazarevic D, Lipovich L, Liu J, Liuni S, McWilliam S, Madan Babu M, Madera M, Marchionni L, Matsuda H, Matsuzawa S, Miki H, Mignone F, Miyake S, Morris K, Mottagui-Tabar S, Mulder N, Nakano N, Nakauchi H, Ng P, Nilsson R, Nishiguchi S, Nishikawa S, Nori F, Ohara O, Okazaki Y, Orlando V, Pang KC, Pavan WJ, Pavesi G, Pesole G, Petrovsky N, Piazza S, Reed J, Reid JF, Ring BZ, Ringwald M, Rost B, Ruan Y, Salzberg SL, Sandelin A, Schneider C, Schonbach C, Sekiguchi K, Semple CA, Seno S, Sessa L, Sheng Y, Shibata Y, Shimada H, Shimada K, Silva D, Sinclair B, Sperling S, Stupka E, Sugiura K, Sultana R, Takenaka Y, Taki K, Tammoja K, Tan SL, Tang S, Taylor MS, Tegner J, Teichmann SA, Ueda HR, van Nimwegen E, Verardo R, Wei CL, Yagi K, Yamanishi H, Zabarovsky E, Zhu S, Zimmer A, Hide W, Bult C, Grimmond SM, Teasdale RD, Liu ET, Brusic V, Quackenbush J, Wahlestedt C, Mattick JS, Hume DA, Kai C, Sasaki D, Tomaru Y, Fukuda S, Kanamori-Katayama M, Suzuki M, Aoki J, Arakawa T, Iida J, Imamura K, Itoh M, Kato T, Kawaji H, Kawagashira N, Kawashima T, Kojima M, Kondo S, Konno H, Nakano K, Ninomiya N, Nishio T, Okada M, Plessy C, Shibata K, Shiraki T, Suzuki S, Tagami M, Waki K, Watahiki A, Okamura-Oho Y, Suzuki H, Kawai J, Hayashizaki Y (2005) The transcriptional landscape of the mammalian genome. *Science* **309:** 1559-1563

Caterina JJ, Skobe Z, Shi J, Ding Y, Simmer JP, Birkedal-Hansen H, Bartlett JD (2002) Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. *J Biol Chem* **277:** 49598-49604

Caterina JJ, Yamada S, Caterina NC, Longenecker G, Holmback K, Shi J, Yermovsky AE, Engler JA, Birkedal-Hansen H (2000) Inactivating mutation of the mouse tissue inhibitor of metalloproteinases-2(Timp-2) gene alters proMMP-2 activation. *J Biol Chem* **275:** 26416-26422

Celiker M, Ramamurthy N, Xu J, Wang M, Jiang Y, Greenwald R, Shi Y (2002) Inhibition of adjuvant-induced arthritis by systemic tissue inhibitor of metalloproteinases 4 gene delivery. *Arthritis Rheum* **46:** 3361-3368

Celiker MY, Wang M, Atsidaftos E, Liu X, Liu YE, Jiang Y, Valderrama E, Goldberg ID, Shi YE (2001) Inhibition of Wilms' tumor growth by intramuscular administration of tissue inhibitor of metalloproteinases-4 plasmid DNA. *Oncogene* **20:** 4337-4343

Chaillan FA, Rivera S, Marchetti E, Jourquin J, Werb Z, Soloway PD, Khrestchatisky M, Roman FS (2006) Involvement of tissue inhibition of metalloproteinases-1 in learning and memory in mice. *Behav Brain Res* **173:** 191-198

Chen C, Huang X, Sheppard D (2006) ADAM33 is not essential for growth and development and does not modulate allergic asthma in mice. *Mol Cell Biol* **26:** 6950-6956

Chen WY, Abatangelo G (1999) Functions of hyaluronan in wound repair. *Wound Repair Regen* **7:** 79-89

Chesneau V, Becherer JD, Zheng Y, Erdjument-Bromage H, Tempst P, Blobel CP (2003) Catalytic properties of ADAM19. *J Biol Chem* **278:** 22331-22340

Chirco R, Liu XW, Jung KK, Kim HR (2006) Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev* **25:** 99-113

Clark IM, Swingler TE, Sampieri CL, Edwards DR (2008) The regulation of matrix metalloproteinases and their inhibitors. *Int J Biochem Cell Biol* **40:** 1362-1378

Cruz-Munoz W, Sanchez OH, Di Grappa M, English JL, Hill RP, Khokha R (2006) Enhanced metastatic dissemination to multiple organs by melanoma and lymphoma cells in timp-3-/- mice. *Oncogene* **25:** 6489-6496

Daley WP, Peters SB, Larsen M (2008) Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci* **121:** 255-264

Dehal P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol* **3:** e314

Docherty AJ, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJ, Murphy G, Reynolds JJ (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* **318:** 66-69

Dollery CM, McEwan JR, Wang M, Sang QA, Liu YE, Shi YE (1999) TIMP-4 is regulated by vascular injury in rats. *Circ Res* **84:** 498-504

Douglas DA, Shi YE, Sang QA (1997) Computational sequence analysis of the tissue inhibitor of metalloproteinase family. *J Protein Chem* **16:** 237-255

Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC (1998) Matrilysin expression and function in airway epithelium. *J Clin Invest* **102:** 1321-1331

Edwards DR, Handsley MM, Pennington CJ (2008) The ADAM metalloproteinases. *Mol Aspects Med* **29:** 258-289

English J, Kassiri Z, Koskivirta I, Atkinson S, Di Grappa M, Soloway P, Nagase H, Vuorio E, Murphy G, Khokha R (2006) Individual Timp deficiencies differentially impact pro-MMP-2 activation. *J Biol Chem* **281:** 10337-10346

Escámez M, García M, Larcher F, Meana A, Muñoz E, Jorcano J, Del Río M (2004) An in vivo model of wound healing in genetically modified skin-humanized mice. *J Invest Dermatol* **123:** 1182-1191

Fanjul-Fernández M, Folgueras AR, Cabrera S, López-Otín C (2010) Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. *Biochim Biophys Acta* **1803:** 3-19

Fata JE, Leco KJ, Voura EB, Yu HY, Waterhouse P, Murphy G, Moorehead RA, Khokha R (2001) Accelerated apoptosis in the Timp-3-deficient mammary gland. *J Clin Invest* **108:** 831-841

Fedak PW, Smookler DS, Kassiri Z, Ohno N, Leco KJ, Verma S, Mickle DA, Watson KL, Hojilla CV, Cruz W, Weisel RD, Li RK, Khokha R (2004) TIMP-3 deficiency leads to dilated cardiomyopathy. *Circulation* **110:** 2401-2409

Felkin LE, Birks EJ, George R, Wong S, Khaghani A, Yacoub MH, Barton PJ (2006) A quantitative gene expression profile of matrix metalloproteinases (MMPS) and their inhibitors (TIMPS) in the myocardium of patients with deteriorating heart failure requiring left ventricular assist device support. *J Heart Lung Transplant* **25:** 1413-1419

Fernandez-Catalan C, Bode W, Huber R, Turk D, Calvete JJ, Lichte A, Tschesche H, Maskos K (1998) Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor. *EMBO J* **17:** 5238-5248

Fernández C, Moses M (2006) Modulation of angiogenesis by tissue inhibitor of metalloproteinase-4. *Biochem Biophys Res Commun* **345:** 523-529

Fielitz J, Leuschner M, Zurbrügg HR, Hannack B, Pregla R, Hetzer R, Regitz-Zagrosek V (2004) Regulation of matrix metalloproteinases and their inhibitors in the left ventricular myocardium of patients with aortic stenosis. *J Mol Med (Berl)* **82:** 809-820

Gasson JC, Golde DW, Kaufman SE, Westbrook CA, Hewick RM, Kaufman RJ, Wong GG, Temple PA, Leary AC, Brown EL (1985) Molecular characterization and expression of the gene encoding human erythroid-potentiating activity. *Nature* **315:** 768-771

Godenschwege TA, Pohar N, Buchner S, Buchner E (2000) Inflated wings, tissue autolysis and early death in tissue inhibitor of metalloproteinases mutants of Drosophila. *Eur J Cell Biol* **79:** 495-501

Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He CS (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. *Proc Natl Acad Sci U S A* **86:** 8207-8211

Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ (1986) Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. *J Biol Chem* **261:** 6600-6605

Gomis-Rüth FX, Maskos K, Betz M, Bergner A, Huber R, Suzuki K, Yoshida N, Nagase H, Brew K, Bourenkov GP, Bartunik H, Bode W (1997) Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. *Nature* **389:** 77-81

Gordon MK, Hahn RA (2010) Collagens. *Cell Tissue Res* **339:** 247-257

Gray JR, Davies SJ (1996) Marfan syndrome. *J Med Genet* **33:** 403-408

Greene J, Wang M, Liu Y, Raymond L, Rosen C, Shi Y (1996) Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem* **271:** 30375-30380

Groft LL, Muzik H, Rewcastle NB, Johnston RN, Knäuper V, Lafleur MA, Forsyth PA, Edwards DR (2001) Differential expression and localization of TIMP-1 and TIMP-4 in human gliomas. *Br J Cancer* **85:** 55-63

Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A* **48:** 1014-1022

Guo YH, Gao W, Li Q, Li PF, Yao PY, Chen K (2004) Tissue inhibitor of metalloproteinases-4 suppresses vascular smooth muscle cell migration and induces cell apoptosis. *Life Sci* **75:** 2483-2493

Gutiérrez-Fernández A, Fueyo A, Folgueras AR, Garabaya C, Pennington CJ, Pilgrim S, Edwards DR, Holliday DL, Jones JL, Span PN, Sweep FC, Puente XS, López-Otín C (2008) Matrix metalloproteinase-8 functions as a metastasis suppressor through modulation of tumor cell adhesion and invasion. *Cancer Res* **68:** 2755-2763

Hadler-Olsen E, Fadnes B, Sylte I, Uhlin-Hansen L, Winberg JO (2011) Regulation of matrix metalloproteinase activity in health and disease. *FEBS J* **278:** 28-45

Handsley MM, Edwards DR (2005) Metalloproteinases and their inhibitors in tumor angiogenesis. *Int J Cancer* **115:** 849-860

Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lübke T, Lena Illert A, von Figura K, Saftig P (2002) The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Hum Mol Genet* **11:** 2615-2624

Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD (1997) Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* **277:** 2002-2004

Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A (1994) Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* **107 (Pt 9):** 2373-2379

Hayashidani S, Tsutsui H, Ikeuchi M, Shiomi T, Matsusaka H, Kubota T, Imanaka-Yoshida K, Itoh T, Takeshita A (2003) Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction. *Am J Physiol Heart Circ Physiol* **285:** H1229-1235

Hernandez-Barrantes S, Shimura Y, Soloway PD, Sang QA, Fridman R (2001) Differential roles of TIMP-4 and TIMP-2 in pro-MMP-2 activation by MT1-MMP. *Biochem Biophys Res Commun* **281:** 126-130

Hjorten R, Hansen U, Underwood RA, Telfer HE, Fernandes RJ, Krakow D, Sebald E, Wachsmann-Hogiu S, Bruckner P, Jacquet R, Landis WJ, Byers PH, Pace JM (2007) Type XXVII collagen at the transition of cartilage to bone during skeletogenesis. *Bone* **41:** 535-542

Hoffmann FG, Opazo JC, Storz JF (2011) Whole-Genome Duplications Spurred the Functional Diversification of the Globin Gene Superfamily in Vertebrates. *Mol Biol Evol*

Hogan B, Beddington R, Constantini F, Lacy E (1994) *Manipulating the mouse embryo: a laboratory manual*, Second Edition edn.: Cold Spring Harbor Laboratory Press.

Hoit BD, Takeishi Y, Cox MJ, Gabel M, Kirkpatrick D, Walsh RA, Tyagi SC (2002) Remodeling of the left atrium in pacing-induced atrial cardiomyopathy. *Mol Cell Biochem* **238:** 145-150

Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* **99:** 81-92

Horiuchi K, Weskamp G, Lum L, Hammes HP, Cai H, Brodie TA, Ludwig T, Chiusaroli R, Baron R, Preissner KT, Manova K, Blobel CP (2003) Potential role for ADAM15 in pathological neovascularization in mice. *Mol Cell Biol* **23:** 5614-5624

Howard L, Zheng Y, Horrocks M, Maciewicz RA, Blobel C (2001) Catalytic activity of ADAM28. *FEBS Lett* **498:** 82-86

Hu J, Van den Steen PE, Sang QX, Opdenakker G (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* **6:** 480-498

Hunder G, Bloch D, Michel B, Stevens M, Arend W, Calabrese L, Edworthy S, Fauci A, Leavitt R, Lie J (1990) The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. *Arthritis Rheum* **33:** 1122-1128

Hunninghake GM, Cho MH, Tesfaigzi Y, Soto-Quiros ME, Avila L, Lasky-Su J, Stidley C, Melén E, Söderhäll C, Hallberg J, Kull I, Kere J, Svartengren M, Pershagen G, Wickman M, Lange C, Demeo DL, Hersh CP, Klanderman BJ, Raby BA, Sparrow D, Shapiro SD, Silverman EK, Litonjua AA, Weiss ST, Celedón JC (2009) MMP12, lung function, and COPD in high-risk populations. *N Engl J Med* **361:** 2599-2608

Hutchinson KR, Guggilam A, Cismowski MJ, Galantowicz ML, West TA, Stewart JA, Zhang X, Lord KC, Lucchesi PA (2011) Temporal pattern of left ventricular structural and functional remodeling following reversal of volume overload heart failure. *J Appl Physiol* **111:** 1778-1788

Hynes RO (2009) The extracellular matrix: not just pretty fibrils. *Science* **326:** 1216-1219

Hynes RO, Naba A (2012) Overview of the matrisome--an inventory of extracellular matrix constituents and functions. *Cold Spring Harb Perspect Biol* **4:** a004903

Illman SA, Lohi J, Keski-Oja J (2008) Epilysin (MMP-28)--structure, expression and potential functions. *Exp Dermatol* **17:** 897-907

Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, López-Otín C, Krane SM (2004) Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci U S A* **101:** 17192-17197

Iruela-Arispe ML, Hasselaar P, Sage H (1991) Differential expression of extracellular proteins is correlated with angiogenesis in vitro. *Lab Invest* **64:** 174-186

Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S (1997) Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. *J Biol Chem* **272:** 22389-22392

Itoh Y, Seiki M (2006) MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol* **206:** 1-8

Jacobsen J, Visse R, Sørensen HP, Enghild JJ, Brew K, Wewer UM, Nagase H (2008) Catalytic properties of ADAM12 and its domain deletion mutants. *Biochemistry* **47:** 537-547

Jane-Lise S, Corda S, Chassagne C, Rappaport L (2000) The extracellular matrix and the cytoskeleton in heart hypertrophy and failure. *Heart Fail Rev* **5:** 239-250

Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R (2009) Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev* **61:** 198-223

Jaworski DM, Beem-Miller M, Lluri G, Barrantes-Reynolds R (2007) Potential regulatory relationship between the nested gene DDC8 and its host gene tissue inhibitor of metalloproteinase-2. *Physiol Genomics* **28:** 168-178

Jaworski DM, Boone J, Caterina J, Soloway P, Falls WA (2005) Prepulse inhibition and fear-potentiated startle are altered in tissue inhibitor of metalloproteinase-2 (TIMP-2) knockout mice. *Brain Res*

Jaworski DM, Soloway P, Caterina J, Falls WA (2006) Tissue inhibitor of metalloproteinase-2(TIMP-2)-deficient mice display motor deficits. *J Neurobiol* **66:** 82-94

Jiang Y, Wang M, Celiker MY, Liu YE, Sang QX, Goldberg ID, Shi YE (2001) Stimulation of mammary tumorigenesis by systemic tissue inhibitor of matrix metalloproteinase 4 gene delivery. *Cancer Res* **61:** 2365-2370

Joronen K, Salminen H, Glumoff V, Savontaus M, Vuorio E (2000) Temporospatial expression of tissue inhibitors of matrix metalloproteinases-1, -2 and -3 during development, growth and aging of the mouse skeleton. *Histochem Cell Biol* **114:** 157-165

Jourquin J, Tremblay E, Bernard A, Charton G, Chaillan FA, Marchetti E, Roman FS, Soloway PD, Dive V, Yiotakis A, Khrestchatisky M, Rivera S (2005) Tissue inhibitor of metalloproteinases-1 (TIMP-1) modulates neuronal death, axonal plasticity, and learning and memory. *Eur J Neurosci* **22:** 2569-2578

Judge DP, Dietz HC (2005) Marfan's syndrome. *The Lancet* **366:** 1965-1976

Jung KK, Liu XW, Chirco R, Fridman R, Kim HR (2006) Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. *EMBO J* **25:** 3934-3942

Kang KH, Park SY, Rho SB, Lee JH (2008) Tissue inhibitor of metalloproteinases-3 interacts with angiotensin II type 2 receptor and additively inhibits angiogenesis. *Cardiovasc Res* **79:** 150-160

Karlin S, Chen C, Gentles AJ, Cleary M (2002) Associations between human disease genes and overlapping gene groups and multiple amino acid runs. *Proc Natl Acad Sci U S A* **99:** 17008-17013

Kashtan CE (1999) Alport syndrome. An inherited disorder of renal, ocular, and cochlear basement membranes. *Medicine (Baltimore)* **78:** 338-360

Kassim SY, Gharib SA, Mecham BH, Birkland TP, Parks WC, McGuire JK (2007) Individual matrix metalloproteinases control distinct transcriptional responses in airway epithelial cells infected with Pseudomonas aeruginosa. *Infect Immun* **75:** 5640-5650

Kassiri Z, Oudit G, Sanchez O, Dawood F, Mohammed F, Nuttall R, Edwards D, Liu P, Backx P, Khokha R (2005) Combination of tumor necrosis factor-alpha ablation and matrix metalloproteinase inhibition prevents heart failure after pressure overload in tissue inhibitor of metalloproteinase-3 knock-out mice. *Circ Res* **97:** 380-390

Kassner A, Tiedemann K, Notbohm H, Ludwig T, Mörgelin M, Reinhardt DP, Chu ML, Bruckner P, Grässel S (2004) Molecular structure and interaction of recombinant human type XVI collagen. *J Mol Biol* **339:** 835-853

Kelly K, Hutchinson G, Nebenius-Oosthuizen D, Smith AJ, Bartsch JW, Horiuchi K, Rittger

A, Manova K, Docherty AJ, Blobel CP (2005) Metalloprotease-disintegrin ADAM8: expression analysis and targeted deletion in mice. *Dev Dyn* **232:** 221-231

Kennedy AM, Inada M, Krane SM, Christie PT, Harding B, López-Otín C, Sánchez LM, Pannett AA, Dearlove A, Hartley C, Byrne MH, Reed AA, Nesbit MA, Whyte MP, Thakker RV (2005) MMP13 mutation causes spondyloepimetaphyseal dysplasia, Missouri type (SEMD(MO). *J Clin Invest* **115:** 2832-2842

Kiger JA, Natzle JE, Kimbrell DA, Paddy MR, Kleinhesselink K, Green MM (2007) Tissue remodeling during maturation of the Drosophila wing. *Dev Biol* **301:** 178-191

Kilpinen S, Autio R, Ojala K, Iljin K, Bucher E, Sara H, Pisto T, Saarela M, Skotheim RI, Björkman M, Mpindi JP, Haapa-Paananen S, Vainio P, Edgren H, Wolf M, Astola J, Nees M, Hautaniemi S, Kallioniemi O (2008) Systematic bioinformatic analysis of expression levels of 17,330 human genes across 9,783 samples from 175 types of healthy and pathological tissues. *Genome Biol* **9:** R139

Kim JW, Simmer JP, Hart TC, Hart PS, Ramaswami MD, Bartlett JD, Hu JC (2005) MMP-20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta. *J Med Genet* **42:** 271-275

Klein T, Bischoff R (2011) Active metalloproteases of the A Disintegrin and Metalloprotease (ADAM) family: biological function and structure. *J Proteome Res* **10:** 17-33

Knäuper V, Will H, López-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G (1996) Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem* **271:** 17124-17131

Koch M, Veit G, Stricker S, Bhatt P, Kutsch S, Zhou P, Reinders E, Hahn RA, Song R, Burgeson RE, Gerecke DR, Mundlos S, Gordon MK (2006) Expression of type XXIII collagen mRNA and protein. *J Biol Chem* **281:** 21546-21557

Kwan JA, Schulze CJ, Wang W, Leon H, Sariahmetoglu M, Sung M, Sawicka J, Sims DE, Sawicki G, Schulz R (2004) Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly (ADP-ribose) polymerase (PARP) in vitro. *FASEB J* **18:** 690-692

Lafleur MA, Handsley MM, Knauper V, Murphy G, Edwards DR (2002) Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). *J Cell Sci* **115:** 3427-3438

Lambert E, Dassé E, Haye B, Petitfrère E (2004) TIMPs as multifacial proteins. *Crit Rev Oncol Hematol* **49:** 187-198

Lausch E, Keppler R, Hilbert K, Cormier-Daire V, Nikkel S, Nishimura G, Unger S, Spranger J, Superti-Furga A, Zabel B (2009) Mutations in MMP9 and MMP13 determine the mode of inheritance and the clinical spectrum of metaphyseal anadysplasia. *Am J Hum Genet* **85:** 168-178

Leco KJ, Apte SS, Taniguchi GT, Hawkes SP, Khokha R, Schultz GA, Edwards DR (1997) Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. *FEBS Lett* **401:** 213-217

Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, Mak TW, Khokha R (2001) Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest* **108:** 817-829

Lee M, Rapti M, Murphy G (2005a) Total conversion of tissue inhibitor of metalloproteinase (TIMP) for specific metalloproteinase targeting: fine-tuning TIMP-4 for optimal inhibition of tumor necrosis factor-{alpha}-converting enzyme. *J Biol Chem* **280:** 15967-15975

Lee MM, Yoon BJ, Osiewicz K, Preston M, Bundy B, van Heeckeren AM, Werb Z, Soloway PD (2005b) Tissue inhibitor of metalloproteinase 1 regulates resistance to infection. *Infect Immun* **73:** 661-665

Li H, Simon H, Bocan TM, Peterson JT (2000) MMP/TIMP expression in spontaneously hypertensive heart failure rats: the effect of ACE- and MMP-inhibition. *Cardiovasc Res* **46:** 298-306

Li YY, Feldman AM, Sun Y, McTiernan CF (1998) Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. *Circulation* **98:** 1728-1734

Li YY, McTiernan CF, Feldman AM (1999) Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. *Cardiovasc Res* **42:** 162-172

Li Z, Clarke MP, Barker MD, McKie N (2005) TIMP3 mutation in Sorsby's fundus dystrophy: molecular insights. *Expert Rev Mol Med* **7:** 1-15

Lijnen HR, Van Hoef B, Vanlinthout I, Verstreken M, Rio MC, Collen D (1999) Accelerated neointima formation after vascular injury in mice with stromelysin-3 (MMP-11) gene inactivation. *Arterioscler Thromb Vasc Biol* **19:** 2863-2870

Liu Y, Wang M, Greene J, Su J, Ullrich S, Li H, Sheng S, Alexander P, Sang Q, Shi Y (1997) Preparation

and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4). *J Biol Chem* **272:** 20479-20483

Lluri G, Langlois GD, McClellan B, Soloway PD, Jaworski DM (2006) Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates neuromuscular junction development via a beta1 integrin-mediated mechanism. *J Neurobiol* **66:** 1365-1377

Lluri G, Langlois GD, Soloway PD, Jaworski DM (2008) Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates myogenesis and beta1 integrin expression in vitro. *Exp Cell Res* **314:** 11-24

Loechel F, Overgaard MT, Oxvig C, Albrechtsen R, Wewer UM (1999) Regulation of human ADAM 12 protease by the prodomain. Evidence for a functional cysteine switch. *J Biol Chem* **274:** 13427-13433

Lohi J, Wilson CL, Roby JD, Parks WC (2001) Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. *J Biol Chem* **276:** 10134-10144

Lovelock JD, Baker AH, Gao F, Dong JF, Bergeron AL, McPheat W, Sivasubramanian N, Mann DL (2005) Heterogeneous effects of tissue inhibitors of matrix metalloproteinases on cardiac fibroblasts. *Am J Physiol Heart Circ Physiol* **288:** H461-468

Lu P, Weaver VM, Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* **196:** 395-406

Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, Keski-Oja J, Wang Y, Skerrett SJ, Parks WC (2009) Epilysin (MMP-28) restrains early macrophage recruitment in Pseudomonas aeruginosa pneumonia. *J Immunol* **182:** 3866-3876

Marchenko GN, Marchenko ND, Strongin AY (2003) The structure and regulation of the human and mouse matrix metalloproteinase-21 gene and protein. *Biochem J* **372:** 503-515

Maretzky T, Yang G, Ouerfelli O, Overall CM, Worpenberg S, Hassiepen U, Eder J, Blobel CP (2009) Characterization of the catalytic activity of the membrane-anchored metalloproteinase ADAM15 in cell-based assays. *Biochem J* **420:** 105-113

Maroteaux P, Verloes A, Stanescu V, Stanescu R (1991) Metaphyseal anadysplasia: a metaphyseal dysplasia of early onset with radiological regression and benign course. *Am J Med Genet* **39:** 4-10

Martignetti JA, Aqeel AA, Sewairi WA, Boumah CE, Kambouris M, Mayouf SA, Sheth KV, Eid WA, Dowling O, Harris J, Glucksman MJ, Bahabri S, Meyer BF, Desnick RJ (2001) Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nat Genet* **28:** 261-265

Matrisian LM, Glaichenhaus N, Gesnel MC, Breathnach R (1985) Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. *EMBO J* **4:** 1435-1440

Matsumura S, Iwanaga S, Mochizuki S, Okamoto H, Ogawa S, Okada Y (2005) Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. *J Clin Invest* **115:** 599-609

Mayers I, Hurst T, Puttagunta L, Radomski A, Mycyk T, Sawicki G, Johnson D, Radomski MW (2001) Cardiac surgery increases the activity of matrix metalloproteinases and nitric oxide synthase in human hearts. *J Thorac Cardiovasc Surg* **122:** 746-752

McCarty M, Baker C, Bucana C, Fidler I (2002) Quantitative and qualitative in vivo angiogenesis assay. *Int J Oncol* **21:** 5-10

Melendez-Zajgla J, Del Pozo L, Ceballos G, Maldonado V (2008) Tissue inhibitor of metalloproteinases-4. The road less traveled. *Mol Cancer* **7:** 85

Mochizuki S, Shimoda M, Shiomi T, Fujii Y, Okada Y (2004) ADAM28 is activated by MMP-7 (matrilysin-1) and cleaves insulin-like growth factor binding protein-3. *Biochem Biophys Res Commun* **315:** 79-84

Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, Ruther U, Edwards DR, Khokha R (2005) Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. *Hepatology* **41:** 857-867

Mohammed FF, Smookler DS, Taylor SE, Fingleton B, Kassiri Z, Sanchez OH, English JL, Matrisian LM, Au B, Yeh WC, Khokha R (2004) Abnormal TNF activity in Timp3-/- mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* **36:** 969-977

Morrison CJ, Butler GS, Bigg HF, Roberts CR, Soloway PD, Overall CM (2001) Cellular activation of MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2-independent pathway. *J Biol Chem* **276:** 47402-47410

Mosig RA, Dowling O, DiFeo A, Ramirez MC, Parker IC, Abe E, Diouri J, Aqeel AA, Wylie JD, Oblander SA, Madri J, Bianco P, Apte SS, Zaidi M, Doty SB, Majeska RJ, Schaffler MB, Martignetti JA (2007) Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in

osteoblast and osteoclast growth. *Hum Mol Genet* **16:** 1113-1123

Mukherjee R, Brinsa TA, Dowdy KB, Scott AA, Baskin JM, Deschamps AM, Lowry AS, Escobar GP, Lucas DG, Yarbrough WM, Zile MR, Spinale FG (2003) Myocardial infarct expansion and matrix metalloproteinase inhibition. *Circulation* **107:** 618-625

Mukherjee R, Herron AR, Lowry AS, Stroud RE, Stroud MR, Wharton JM, Ikonomidis JS, Crumbley AJ, Spinale FG, Gold MR (2006) Selective induction of matrix metalloproteinases and tissue inhibitor of metalloproteinases in atrial and ventricular myocardium in patients with atrial fibrillation. *Am J Cardiol* **97:** 532-537

Muraguchi T, Takegami Y, Ohtsuka T, Kitajima S, Chandana EP, Omura A, Miki T, Takahashi R, Matsumoto N, Ludwig A, Noda M, Takahashi C (2007) RECK modulates Notch signaling during cortical neurogenesis by regulating ADAM10 activity. *Nat Neurosci* **10:** 838-845

Murphy G, Knäuper V (1997) Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? *Matrix Biol* **15:** 511-518

Murphy G, Nguyen Q, Cockett MI, Atkinson SJ, Allan JA, Knight CG, Willenbrock F, Docherty AJ (1994) Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. *J Biol Chem* **269:** 6632-6636

Murphy G, Segain JP, O'Shea M, Cockett M, Ioannou C, Lefebvre O, Chambon P, Basset P (1993) The 28-kDa N-terminal domain of mouse stromelysin-3 has the general properties of a weak metalloproteinase. *J Biol Chem* **268:** 15435-15441

Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. *Ann Med* **33:** 7-21

Myllyharju J, Kivirikko KI (2004) Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet* **20:** 33-43

Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* **69:** 562-573

Newby AC (2012) Matrix metalloproteinase inhibition therapy for vascular diseases. *Vascul Pharmacol*

Nothnick WB (2000) Disruption of the tissue inhibitor of metalloproteinase-1 gene results in altered reproductive cyclicity and uterine morphology in reproductive-age female mice. *Biol Reprod* **63:** 905-912

Nothnick WB (2001) Reduction in reproductive lifespan of tissue inhibitor of metalloproteinase 1 (TIMP-1)-deficient female mice. *Reproduction* **122:** 923-927

Nuttall RK, Sampieri CL, Pennington CJ, Gill SE, Schultz GA, Edwards DR (2004) Expression analysis of the entire MMP and TIMP gene families during mouse tissue development. *FEBS Lett* **563:** 129-134

Oh J, Seo DW, Diaz T, Wei B, Ward Y, Ray JM, Morioka Y, Shi S, Kitayama H, Takahashi C, Noda M, Stetler-Stevenson WG (2004a) Tissue inhibitors of metalloproteinase 2 inhibits endothelial cell migration through increased expression of RECK. *Cancer Res* **64:** 9062-9069

Oh J, Takahashi R, Adachi E, Kondo S, Kuratomi S, Noma A, Alexander DB, Motoda H, Okada A, Seiki M, Itoh T, Itohara S, Takahashi C, Noda M (2004b) Mutations in two matrix metalloproteinase genes, MMP-2 and MT1-MMP, are synthetic lethal in mice. *Oncogene* **23:** 5041-5048

Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C, Noda M (2001) The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* **107:** 789-800

Ohta K, Nakajima T, Cheah AY, Zaidi SH, Kaviani N, Dawood F, You XM, Liu P, Husain M, Rabinovitch M (2004) Elafin-overexpressing mice have improved cardiac function after myocardial infarction. *Am J Physiol Heart Circ Physiol* **287:** H286-292

Oksjoki S, Sallinen S, Vuorio E, Anttila L (1999) Cyclic expression of mRNA transcripts for connective tissue components in the mouse ovary. *Mol Hum Reprod* **5:** 803-808

Olson T, Hirohata S, Ye J, Leco K, Seldin M, Apte S (1998) Cloning of the human tissue inhibitor of metalloproteinase-4 gene (TIMP4) and localization of the TIMP4 and Timp4 genes to human chromosome 3p25 and mouse chromosome 6, respectively. *Genomics* **51:** 148-151

Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8:** 221-233

Parks WC, Wilson CL, López-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* **4:** 617-629

Parry DA, Toomes C, Bida L, Danciger M, Towns KV, McKibbin M, Jacobson SG, Logan CV, Ali M, Bond J, Chance R, Swendeman S, Daniele LL, Springell K, Adams M, Johnson CA, Booth AP, Jafri H,

Rashid Y, Banin E, Strom TM, Farber DB, Sharon D, Blobel CP, Pugh EN, Pierce EA, Inglehearn CF (2009) Loss of the metalloprotease ADAM9 leads to cone-rod dystrophy in humans and retinal degeneration in mice. *Am J Hum Genet* **84:** 683-691

Pavloff N, Staskus PW, Kishnani NS, Hawkes SP (1992) A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. *J Biol Chem* **267:** 17321-17326

Pei D, Kang T, Qi H (2000) Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. *J Biol Chem* **275:** 33988-33997

Pei D, Weiss SJ (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* **375:** 244-247

Peterson JT (2006) The importance of estimating the therapeutic index in the development of matrix metalloproteinase inhibitors. *Cardiovasc Res* **69:** 677-687

Peterson JT, Hallak H, Johnson L, Li H, O'Brien PM, Sliskovic DR, Bocan TM, Coker ML, Etoh T, Spinale FG (2001) Matrix metalloproteinase inhibition attenuates left ventricular remodeling and dysfunction in a rat model of progressive heart failure. *Circulation* **103:** 2303-2309

Peterson JT, Li H, Dillon L, Bryant JW (2000) Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. *Cardiovasc Res* **46:** 307-315

Pilka R, Domanski H, Hansson S, Eriksson P, Casslén B (2004) Endometrial TIMP-4 mRNA is high at midcycle and in hyperplasia, but down-regulated in malignant tumours. Coordinated expression with MMP-26. *Mol Hum Reprod* **10:** 641-650

Pilka R, Noskova V, Domanski H, Andersson C, Hansson S, Casslén B (2006) Endometrial TIMP-4 mRNA is expressed in the stroma, while TIMP-4 protein accumulates in the epithelium and is released to the uterine fluid. *Mol Hum Reprod* **12:** 497-503

Pohar N, Godenschwege TA, Buchner E (1999) Invertebrate tissue inhibitor of metalloproteinase: structure and nested gene organization within the synapsin locus is conserved from Drosophila to human. *Genomics* **57:** 293-296

Porter S, Clark IM, Kevorkian L, Edwards DR (2005) The ADAMTS metalloproteinases. *Biochem J* **386:** 15-27

Prockop DJ, Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* **64:** 403-434

Pruitt KD, Tatusova T, Maglott DR (2007) NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **35:** D61-65

Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, Baker A, Anand-Apte B (2003) A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nat Med* **9:** 407-415

Ra HJ, Parks WC (2007) Control of matrix metalloproteinase catalytic activity. *Matrix Biol* **26:** 587-596

Radomski A, Jurasz P, Sanders E, Overall C, Bigg H, Edwards D, Radomski M (2002) Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets. *Br J Pharmacol* **137:** 1330-1338

Rawlings ND, Barrett AJ, Bateman A (2012) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res* **40:** D343-350

Roten L, Nemoto S, Simsic J, Coker ML, Rao V, Baicu S, Defreyte G, Soloway PJ, Zile MR, Spinale FG (2000) Effects of gene deletion of the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1) on left ventricular geometry and function in mice. *J Mol Cell Cardiol* **32:** 109-120

Rouzier C, Vanatka R, Bannwarth S, Philip N, Coussement A, Paquis-Flucklinger V, Lambert JC (2006) A novel homozygous MMP2 mutation in a family with Winchester syndrome. *Clin Genet* **69:** 271-276

Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA (2004) ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. *J Biol Chem* **279:** 51323-51330

Sahebjam S, Khokha R, Mort JS (2007) Increased collagen and aggrecan degradation with age in the joints of Timp3(-/-) mice. *Arthritis Rheum* **56:** 905-909

Schaefer L, Iozzo RV (2008) Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J Biol Chem* **283:** 21305-21309

Schulze CJ, Wang W, Suarez-Pinzon WL, Sawicka J, Sawicki G, Schulz R (2003) Imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial

[correction of myoctardial] ischemia-reperfusion injury. *Circulation* **107:** 2487-2492

Seeland U, Kouchi I, Zolk O, Itter G, Linz W, Bohm M (2002) Effect of ramipril and furosemide treatment on interstitial remodeling in post-infarction heart failure rat hearts. *J Mol Cell Cardiol* **34:** 151-163

Seiki M (2003) Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Lett* **194:** 1-11

Seo DW, Li H, Guedez L, Wingfield PT, Diaz T, Salloum R, Wei BY, Stetler-Stevenson WG (2003) TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. *Cell* **114:** 171-180

Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD (1996) Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc Natl Acad Sci U S A* **93:** 3942-3946

Smookler D, Mohammed F, Kassiri Z, Duncan G, Mak T, Khokha R (2006) Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation. *J Immunol* **176:** 721-725

Sohail A, Sun Q, Zhao H, Bernardo MM, Cho JA, Fridman R (2008) MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer. *Cancer Metastasis Rev* **27:** 289-302

Soloway PD, Alexander CM, Werb Z, Jaenisch R (1996) Targeted mutagenesis of Timp-1 reveals that lung tumor invasion is influenced by Timp-1 genotype of the tumor but not by that of the host. *Oncogene* **13:** 2307-2314

Staskus PW, Masiarz FR, Pallanck LJ, Hawkes SP (1991) The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. *J Biol Chem* **266:** 449-454

Stetler-Stevenson WG, Bersch N, Golde DW (1992) Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. *FEBS Lett* **296:** 231-234

Stetler-Stevenson WG, Krutzsch HC, Liotta LA (1989) Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. *J Biol Chem* **264:** 17374-17378

Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z (2004) Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* **131:** 5883-5895

Stracke JO, Fosang AJ, Last K, Mercuri FA, Pendás AM, Llano E, Perris R, Di Cesare PE, Murphy G, Knäuper V (2000a) Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP). *FEBS Lett* **478:** 52-56

Stracke JO, Hutton M, Stewart M, Pendas AM, Smith B, Lopez-Otin C, Murphy G, Knauper V (2000b) Biochemical characterization of the catalytic domain of human matrix metalloproteinase 19. Evidence for a role as a potent basement membrane degrading enzyme. *J Biol Chem* **275:** 14809-14816

Stratmann B, Farr M, Tschesche H (2001) Characterization of C-terminally truncated human tissue inhibitor of metalloproteinases-4 expressed in Pichia pastoris. *Biol Chem* **382:** 987-991

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* **270:** 5331-5338

Su J, Gorse K, Ramirez F, Fox MA (2010) Collagen XIX is expressed by interneurons and contributes to the formation of hippocampal synapses. *J Comp Neurol* **518:** 229-253

Tallant C, Marrero A, Gomis-Rüth FX (2010) Matrix metalloproteinases: fold and function of their catalytic domains. *Biochim Biophys Acta* **1803:** 20-28

Talts J, Brakebusch C, Fässler R (1999) Integrin gene targeting. *Methods Mol Biol* **129:** 153-187

Tao ZY, Cavasin MA, Yang F, Liu YH, Yang XP (2004) Temporal changes in matrix metalloproteinase expression and inflammatory response associated with cardiac rupture after myocardial infarction in mice. *Life Sci* **74:** 1561-1572

Tong Y, Xu Y, Scearce-Levie K, Ptácek LJ, Fu YH (2010) COL25A1 triggers and promotes Alzheimer's disease-like pathology in vivo. *Neurogenetics* **11:** 41-52

Troeberg L, Fushimi K, Khokha R, Emonard H, Ghosh P, Nagase H (2008) Calcium pentosan polysulfate is a multifaceted exosite inhibitor of aggrecanases. *FASEB J* **22:** 3515-3524

Troeberg L, Tanaka M, Wait R, Shi YE, Brew K, Nagase H (2002) E. coli expression of TIMP-4 and comparative kinetic studies with TIMP-1 and TIMP-2: insights into the interactions of TIMPs and matrix metalloproteinase 2 (gelatinase A). *Biochemistry* **41:** 15025-15035

Tummalapalli CM, Heath BJ, Tyagi SC (2001) Tissue inhibitor of metalloproteinase-4 instigates apoptosis in transformed cardiac fibroblasts. *J Cell Biochem* **80:** 512-521

Tunuguntla R, Ripley D, Sang QX, Chegini N (2003) Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinases TIMP-3 and -4 in benign endometrium and endometrial cancer. *Gynecol Oncol* **89:** 453-459

Uría JA, López-Otín C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. *Cancer Res* **60:** 4745-4751

Vaalamo M, Leivo T, Saarialho-Kere U (1999) Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) in normal and aberrant wound healing. *Hum Pathol* **30:** 795-802

Vanhoutte D, Schellings M, Pinto Y, Heymans S (2006) Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. *Cardiovasc Res* **69:** 604-613

Vater CA, Mainardi CL, Harris ED (1979) Inhibitor of human collagenase from cultures of human tendon. *J Biol Chem* **254:** 3045-3053

Veit G, Kobbe B, Keene DR, Paulsson M, Koch M, Wagener R (2006) Collagen XXVIII, a novel von Willebrand factor A domain-containing protein with many imperfections in the collagenous domain. *J Biol Chem* **281:** 3494-3504

Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* **92:** 827-839

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* **93:** 411-422

Wagenseil JE, Mecham RP (2009) Vascular extracellular matrix and arterial mechanics. *Physiol Rev* **89:** 957-989

Wang M, Liu Y, Greene J, Sheng S, Fuchs A, Rosen E, Shi Y (1997) Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. *Oncogene* **14:** 2767-2774

Wang T, Yamashita K, Iwata K, Hayakawa T (2002) Both tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 activate Ras but through different pathways. *Biochem Biophys Res Commun* **296:** 201-205

Wang Z, Juttermann R, Soloway PD (2000) TIMP-2 is required for efficient activation of proMMP-2 in vivo. *J Biol Chem* **275:** 26411-26415

Weber BH, Lin B, White K, Kohler K, Soboleva G, Herterich S, Seeliger MW, Jaissle GB, Grimm C, Reme C, Wenzel A, Asan E, Schrewe H (2002) A mouse model for Sorsby fundus dystrophy. *Invest Ophthalmol Vis Sci* **43:** 2732-2740

Wei P, Zhao YG, Zhuang L, Ruben S, Sang QX (2001) Expression and enzymatic activity of human disintegrin and metalloproteinase ADAM19/meltrin beta. *Biochem Biophys Res Commun* **280:** 744-755

Wei S, Xie Z, Filenova E, Brew K (2003) Drosophila TIMP is a potent inhibitor of MMPs and TACE: similarities in structure and function to TIMP-3. *Biochemistry* **42:** 12200-12207

Welgus HG, Stricklin GP, Eisen AZ, Bauer EA, Cooney RV, Jeffrey JJ (1979) A specific inhibitor of vertebrate collagenase produced by human skin fibroblasts. *J Biol Chem* **254:** 1938-1943

Weskamp G, Cai H, Brodie TA, Higashyama S, Manova K, Ludwig T, Blobel CP (2002) Mice lacking the metalloprotease-disintegrin MDC9 (ADAM9) have no evident major abnormalities during development or adult life. *Mol Cell Biol* **22:** 1537-1544

Wilson EM, Moainie SL, Baskin JM, Lowry AS, Deschamps AM, Mukherjee R, Guy TS, St John-Sutton MG, Gorman JH, 3rd, Edmunds LH, Jr., Gorman RC, Spinale FG (2003) Region- and type-specific induction of matrix metalloproteinases in post-myocardial infarction remodeling. *Circulation* **107:** 2857-2863

Wiseman BS, Sternlicht MD, Lund LR, Alexander CM, Mott J, Bissell MJ, Soloway P, Itohara S, Werb Z (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *J Cell Biol* **162:** 1123-1133

Woolley DE, Glanville RW, Crossley MJ, Evanson JM (1975) Purification of rheumatoid synovial collagenase and its action on soluble and insoluble collagen. *Eur J Biochem* **54:** 611-622

Yana I, Seiki M (2002) MT-MMPs play pivotal roles in cancer dissemination. *Clin Exp Metastasis* **19:** 209-215

Yang M, Kurkinen M (1998) Cloning and characterization of a novel matrix metalloproteinase (MMP), CMMP, from chicken embryo fibroblasts. CMMP, Xenopus XMMP, and human MMP19 have a conserved unique cysteine in the catalytic domain. *J Biol Chem* **273:** 17893-17900

Yang P, Baker KA, Hagg T (2005) A disintegrin and metalloprotease 21 (ADAM21) is associated with neurogenesis and axonal growth in developing and adult rodent CNS. *J Comp Neurol* **490:** 163-179

Yi C, Woo JM, Han C, Oh JS, Park I, Lee B, Jin S, Choi H, Kwon JT, Cho BN, Kim dH, Cho C (2010) Expression analysis of the Adam21 gene in mouse testis. *Gene Expr Patterns* **10:** 152-158

Young DA, Phillips BW, Lundy C, Nuttall RK, Hogan A, Schultz GA, Leco KJ, Clark IM, Edwards DR (2002) Identification of an initiator-like element essential for the expression of the tissue inhibitor of metalloproteinases-4 (Timp-4) gene. *Biochem J* **364:** 89-99

Yu WH, Yu S, Meng Q, Brew K, Woessner JF, Jr. (2000) TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. *J Biol Chem* **275:** 31226-31232

Zankl A, Bonafé L, Calcaterra V, Di Rocco M, Superti-Furga A (2005) Winchester syndrome caused by a homozygous mutation affecting the active site of matrix metalloproteinase 2. *Clin Genet* **67:** 261-266

Zankl A, Pachman L, Poznanski A, Bonafé L, Wang F, Shusterman Y, Fishman DA, Superti-Furga A (2007) Torg syndrome is caused by inactivating mutations in MMP2 and is allelic to NAO and Winchester syndrome. *J Bone Miner Res* **22:** 329-333

Zhang J, Cao YJ, Zhao YG, Sang QX, Duan EK (2002) Expression of matrix metalloproteinase-26 and tissue inhibitor of metalloproteinase-4 in human normal cytotrophoblast cells and a choriocarcinoma cell line, JEG-3. *Mol Hum Reprod* **8:** 659-666

Zhang L, Hill RP (2004) Hypoxia enhances metastatic efficiency by up-regulating Mdm2 in KHT cells and increasing resistance to apoptosis. *Cancer Res* **64:** 4180-4189

Zhao H, Bernardo MM, Osenkowski P, Sohail A, Pei D, Nagase H, Kashiwagi M, Soloway PD, DeClerck YA, Fridman R (2004a) Differential inhibition of membrane type 3 (MT3)-matrix metalloproteinase (MMP) and MT1-MMP by tissue inhibitor of metalloproteinase (TIMP)-2 and TIMP-3 rgulates pro-MMP-2 activation. *J Biol Chem* **279:** 8592-8601

Zhao YG, Xiao AZ, Park HI, Newcomer RG, Yan M, Man YG, Heffelfinger SC, Sang QX (2004b) Endometase/matrilysin-2 in human breast ductal carcinoma in situ and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: a putative role in the initiation of breast cancer invasion. *Cancer Res* **64:** 590-598

Zhou HM, Weskamp G, Chesneau V, Sahin U, Vortkamp A, Horiuchi K, Chiusaroli R, Hahn R, Wilkes D, Fisher P, Baron R, Manova K, Basson CT, Hempstead B, Blobel CP (2004) Essential role for ADAM19 in cardiovascular morphogenesis. *Mol Cell Biol* **24:** 96-104

Zou J, Zhu F, Liu J, Wang W, Zhang R, Garlisi C, Liu Y, Wang S, Shah H, Wan Y, Umland S (2004) Catalytic activity of human ADAM33. *J Biol Chem* **279:** 9818-9830